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Review Article

Serine proteases as targets for antithrombotic therapy

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CONTENTS

Abstract

Pathological activation of the hemostatic system, resulting in thromboembolic complications, is a major cause of morbidity and mortality. Unfractionated heparins and low-molecular-weight heparins, both suitable only for parenteral administration, as well as vitamin K antagonists, represent the current cornerstone of antithrombotic therapy. Although orally active and very efficacious, the latter class of drugs has a narrow therapeutic window and requires frequent monitoring. Due to their apparent pivotal role in the coagulation cascade, the serine proteases factor Xa and thrombin have attracted the greatest attention in thrombosis research. The recently launched fondaparinux, an antithrombin-III-dependent pentasaccharide and highly selective factor Xa inhibitor, has demonstrated impressive clinical results and may play an important role in the future. On the other hand, the search for low-molecular-weight, orally active direct serine protease inhibitors has been the focus of intense research for more than a decade. However, only a very limited number of compounds are under late clinical development to date. The most advanced compound, the orally active, selective thrombin inhibitor ximelagatran, is in the preregistration phase for the treatment of deep vein thrombosis. Factor VIIa and factor IXa have gained less attention as pharmacological targets, factor XIa and factor XIIa play only a minor role in thrombosis research.

Introduction

Cardiovascular diseases are the major cause of death and disabilities in Western societies. The formation of an occlusive blood clot is the result of disturbances of normal blood flow, excessive activation of platelets and initiation of the coagulation cascade. Antithrombotic therapy usually consists of a combination of anticoagulant and antiplatelet agents, *e.g*., heparin and aspirin. In addition, the vitamin K antagonists are used as oral anticoagulants, although they exert a narrow therapeutic window, which makes clinical monitoring necessary.

Intense research efforts have been concentrated on the principle mechanisms involved in thrombosis and several new biological targets have been identified which may be useful for therapeutic intervention (1). New therapeutic principles aiming at the control of platelet activation and aggregation are fibrinogen receptor antagonists, ADP receptor antagonists and combined thromboxane A_2 synthase inhibitors/receptor antagonists. The serine proteases of the coagulation cascade such as thrombin and factor Xa have emerged as attractive targets for antithrombotic intervention. In addition, von Willebrand factor, P-selectin or plasminogen activator inhibitor-1 are considered as possible targets for antithrombotic intervention.

This review concentrates on the serine proteases involved in the coagulation cascade and describes their biological roles and the general principles to control their biological activities. The most important inhibitors of these serine proteases will be described, along with data concerning their biological effects and developmental status.

Serine proteases in the coagulation cascade

The hemostatic system is responsible for the prevention of excessive blood loss and restoration of normal blood flow following tissue or vascular injury, which is a necessary prerequisite for survival in highly developed species.

The coagulation cascade (Fig. 1) is a proteolytic cascade involving several serine proteases, leading to

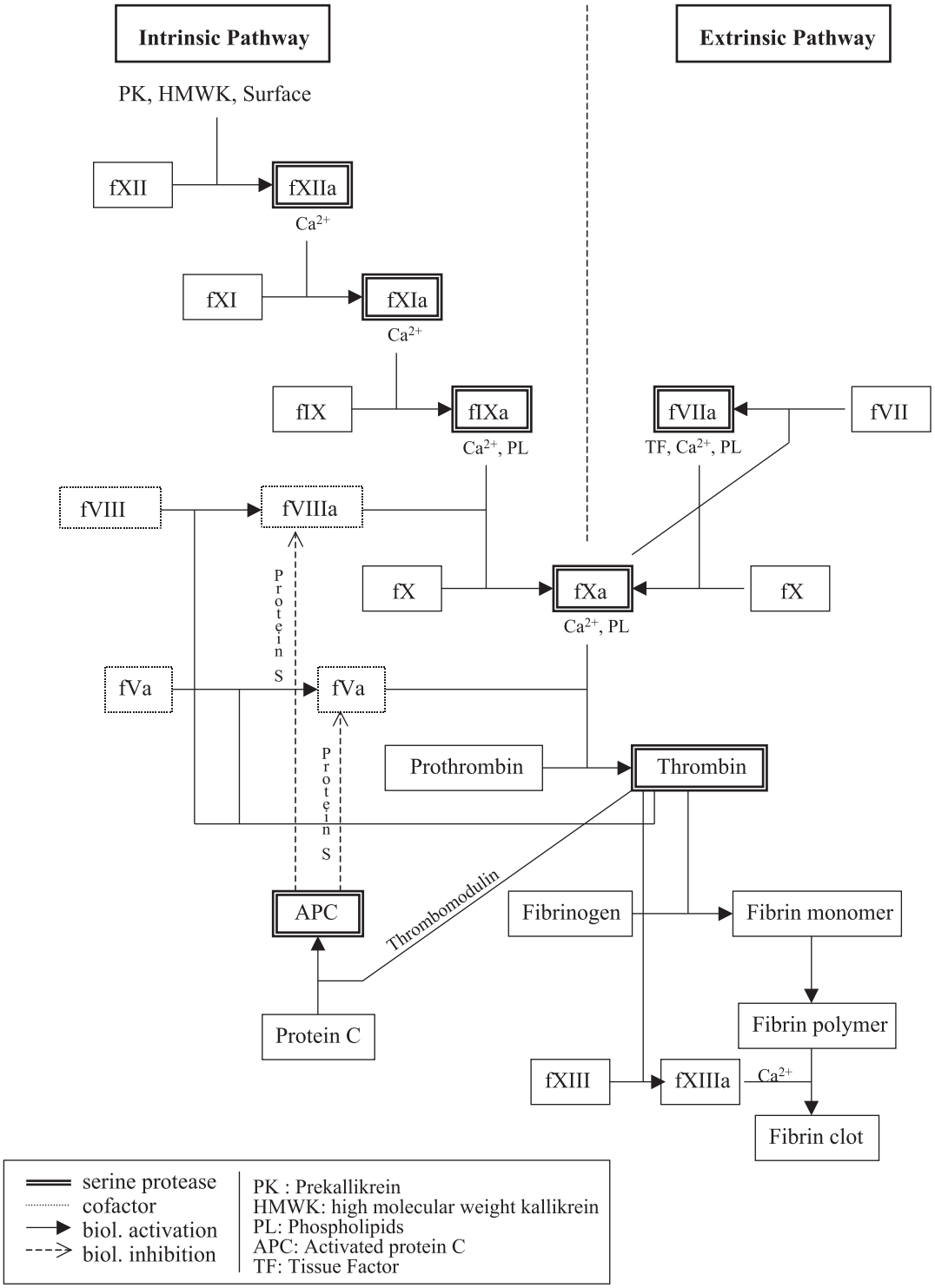

Fig. 1. Blood coagulation cascade.

hemostasis. Each enzyme of the pathway circulates in the plasma as an inactive precursor zymogen, which upon activation undergoes proteolytic cleavage to release the active factor from the precursor molecule. The coagulation pathway functions as a series of positive and negative feedback loops, which control the activation process, leading to the formation of active thrombin. Thrombin, in turn, converts soluble fibrinogen into fibrin, which forms a clot. The coagulation cascade is divided into three phases: the intrinsic and extrinsic pathways

both providing alternative routes for the generation of factor Xa, and the final common pathway leading to thrombin formation.

The intrinsic pathway, also termed the contact phase of blood coagulation, is activated upon exposure of blood to various foreign surfaces, *e.g.*, glass or plastic surfaces after blood withdrawal or when it comes into contact with subendothelial tissues or with negatively charged surfaces that are exposed as a result of tissue damage. The serine proteases factor XIIa (fXIIa, Hageman factor), factor XIa (fXIa) and factor IXa (fIXa) are involved in this activation process. The first step is the binding of fXII to a subendothelial surface exposed by tissue injury. Prekallikrein and high-molecular-weight kininogen (HMWK), also assembled on this surface in close proximity, interact and subsequently activate fXII. fXIIa remains in close contact with the activating surface and, in the presence of Ca^{2+}, leads to the activation of fXI. fXIa then converts fIX into fIXa, again in a Ca^{2+}-dependent manner. fIXa assembles with the cofactor VIIIa in the presence of Ca^{2+} on the surface of activated platelets leading to the formation of the tenase complex, which is responsible for the activation of factor X (fX), a pivotal serine protease in the coagulation cascade. Within the tenase complex fVIIIa acts as a receptor for factors IXa and X. During coagulation, fVIII is modified by active thrombin, which results in greatly enhanced fVIII activity, promoting the activation of fX.

The extrinsic pathway of coagulation provides a very rapid response to tissue or blood vessel injury, leading almost instantaneously to the generation of activated fX, compared to the seconds or even minutes required for activation of fX via the intrinsic pathway. The principal function of the extrinsic pathway is to augment the activity of the intrinsic pathway.

Two components are unique to the extrinsic pathway, namely tissue factor (TF) and the serine protease factor VIIa. TF is an integral membrane protein abundantly expressed in many human cells and tissues, *e.g.*, on platelets and in the vascular adventitia. Once activated, TF binds rapidly to the serine protease zymogen fVII, which is activated to form a complex with TF, Ca^{2+} and phospholipids. This complex in turn rapidly activates fX.

The intrinsic and extrinsic systems converge at fXa to a single common pathway which is ultimately responsible for the production of the serine protease thrombin (factor IIa). On the surface of activated platelets, fXa, the cofactor Va, Ca^{2+}, prothrombin and platelet phospholipids form the prothrombinase complex. Factor V is activated to fVa by very small amounts of thrombin and is inactivated by increased levels of thrombin. Similar to the role of fVIIIa in the tenase complex, fVa binds to specific receptors on the surfaces of activated platelets and forms a complex with prothrombin and fXa.

Thrombin, generated within the prothrombinase complex, rapidly converts fibrinogen to fibrin, which subsequently polymerizes to form the matrix of the developing blood clot. In addition to its role in the activation of fibrin clot formation, thrombin plays an important regulatory role in coagulation. After binding to thrombomodulin, the substrate specificity of thrombin is changed and protein C becomes its preferred substrate. Activated protein C (aPC) and cofactor protein S degrade factors Va and VIIIa, thereby limiting the process of thrombin generation.

The activity of thrombin is mainly controlled by the serine protease inhibitor antithrombin III (AT-III), which also inhibits the activities of factors IXa, Xa, XIa and XIIa. After binding to heparin the activity of AT-III is greatly enhanced due to a conformational change. This effect of heparin is the basis for its clinical use as an anticoagulant.

Thrombin

The biological precursor of thrombin is prothrombin, which is present in plasma at a concentration of about 1.4 μM (2). As described above, thrombin exhibits a number of important biological activities. In addition, it transforms coagulation factor XIII to factor XIIIa, which covalently cross-links the fibrin strands. Thrombin is responsible for a variety of cellular actions mediated by binding to specific protease-activated receptors (3). In addition, it is one of the most potent stimulators of platelet aggregation and also a potent mitogen for vascular smooth muscle cells.

Due to its multiple physiological actions, thrombin is an ideal target for drug development (1, 4-6). Historically, antithrombotic therapy was mainly based on agents that prevent thrombin formation (*e.g.*, coumarin) or on indirect thrombin inhibitors like unfractionated heparin (UFH). Although both types of drugs are extensively used for the treatment and prophylaxis of thrombosis, they suffer from several disadvantages such as a narrow therapeutic window and a highly variable dose-response relationship, which may result either in significant bleeding risk or insufficient clinical benefit (7, 8). In addition, heparin has to be administered parenterally and is ineffective in patients with AT-III deficiency (9). Treatment with coumarin or heparin requires frequent monitoring in order to adjust for the clinically relevant antithrombotic dose and to prevent hemorrhagic complications.

The modern low-molecular-weight heparins (LMWH) show improved pharmacokinetic profiles and more reliable dose-responses, and thus are administered subcutaneously without monitoring. However, as with heparin, they do not inhibit clot-bound thrombin and may also cause thrombocytopenia, a severe side effect observed in approx. 1-5% of patients on heparin treatment (10).

In order to overcome these obstacles and to develop improved antithrombotic agents, the search for direct thrombin inhibitors has been pursued during the last two decades. Direct thrombin inhibitors can be divided into two classes: native and synthetic thrombin inhibitors. Native thrombin inhibitors have originated from blood-sucking animals such as leeches or bugs. Hirudin, a 65-amino acid polypeptide, isolated from the medicinal leech *Hirudo medicinalis*, is the most potent specific thrombin

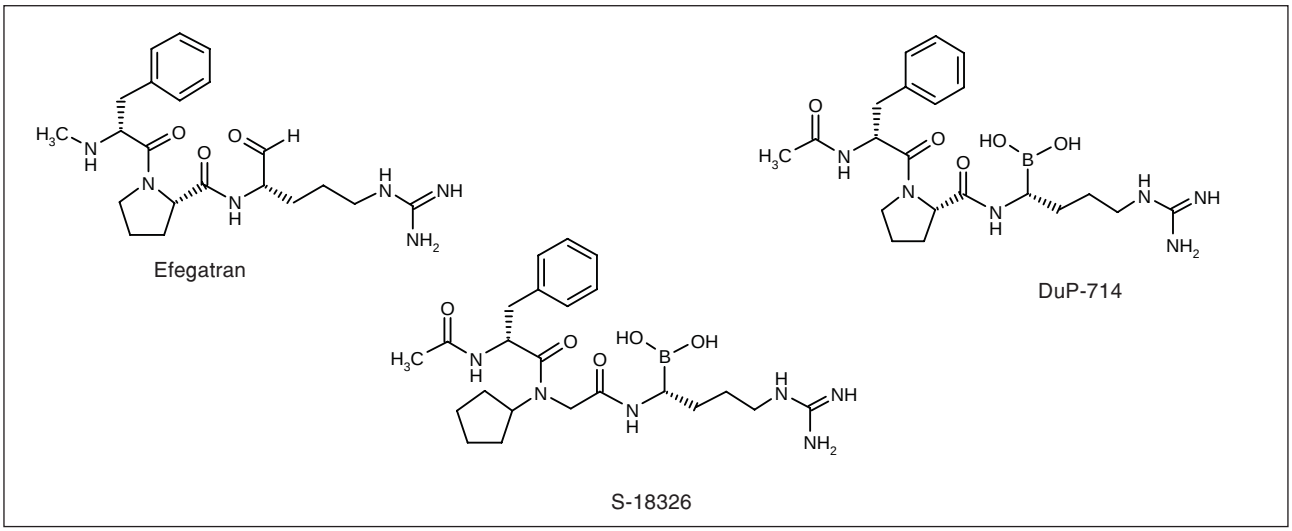

Fig. 2. Transition state thrombin inhibitors.

inhibitor known thus far (K_i = 20 fM) (11). It interacts with the active site and the carboxyterminal exosite 1 of thrombin forming a stoichiometric, slowly reversible complex (12, 13). Hirudin has been registered successfully as replacement therapy for patients with heparin-induced thrombocytopenia, suffering from arterial or venous thrombotic diseases (14, 15). In patients with unstable angina and non-ST-elevation myocardial infarction, hirudin appears to be more effective than heparin (16, 17).

The dodecapeptide analog bivalirudin differs from hirudin by a weaker enzyme inhibition (K_i = 2 nM) (18) and a shorter half-life, which may result in an improved safety. In phase III trials in patients undergoing coronary angioplasty for unstable angina, bivalirudin was at least as effective as high-dose heparin in preventing ischemic complications (19). In 2000 the compound was approved in the U.S. for use in patients undergoing percutaneous transluminal coronary angioplasty (PCTA).

The early synthetic thrombin inhibitors were designed by modifications of the fibrinogen amino acid sequence around the thrombin scissile bond. Most of these active-site directed inhibitors contain the sequence D-Phe-Pro-Arg and a reactive moiety interacting irreversibly with the enzyme. Examples of these "transition state analogs" are the arginine-aldehyde derivative efegatran and the boronic acid derivatives DuP-714 and S-18326 (Fig. 2).

Efegatran is a tight-binding, reversible thrombin inhibitor (K_i = 40 nM) with good selectivity *versus* related serine proteases (20). Upon binding to thrombin, a hemiacetal is formed between the aldehyde carbonyl group of the inhibitor and the OH-group of Ser 195. In rat and rabbit studies of venous and arterial thrombosis, efegatran showed antithrombotic efficacy after both intravenous and oral administration (21). In patients with unstable angina, no clinical benefit compared to heparin was observed (22). In addition, the combination of efegatran plus streptokinase was not superior to the combination of heparin with accelerated t-PA in achieving early patency in patients with acute myocardial infarction (23). To date, the developmental status of efegatran is unclear.

The arginine-boronic acid derivative DuP-714, a subnanomolar (K_i = 0.04 nM) and orally active thrombin inhibitor, showed efficacy in animal models of venous and arterial thrombosis (24). However, at therapeutic doses it caused hypotension, localized inflammation and transient thrombocytopenia, which seemed to be related to inhibition of complement factor I (25). Because of these undesired effects, the development of DuP-714 was terminated.

S-18326 is an analog of DuP-714 with reduced *in vitro* potency (IC_{50} = 4 nM) and an improved selectivity profile (26). This compound showed antithrombotic activity in different animal species after oral administration (27). In phase I clinical trials it has been shown to increase the ratios of several clotting assays linearly with the measured plasma concentration after i.v. administration (28). No clinical results in patients have been reported thus far.

A general point of concern for the feasibility of the transition state analog approach is the slow enzyme inhibition kinetic. It was shown that slow-binding inhibitors like efegatran exhibit steeper dose-response curves resulting in a more narrow therapeutic window compared to reversible, fast-acting inhibitors such as melagatran (see below) (29).

Removal of the serine trap in the early transition state analogs and modifications of lipophilic residues in order to improve the inhibitory potency provided competitive, non-covalent inhibitors with superior biological activity *in vivo*. Compounds selected for in-depth preclinical and clinical evaluation typically contain carboxylate moieties, which counterbalance the positively charged guanidine group. These zwitterionic compounds, such as

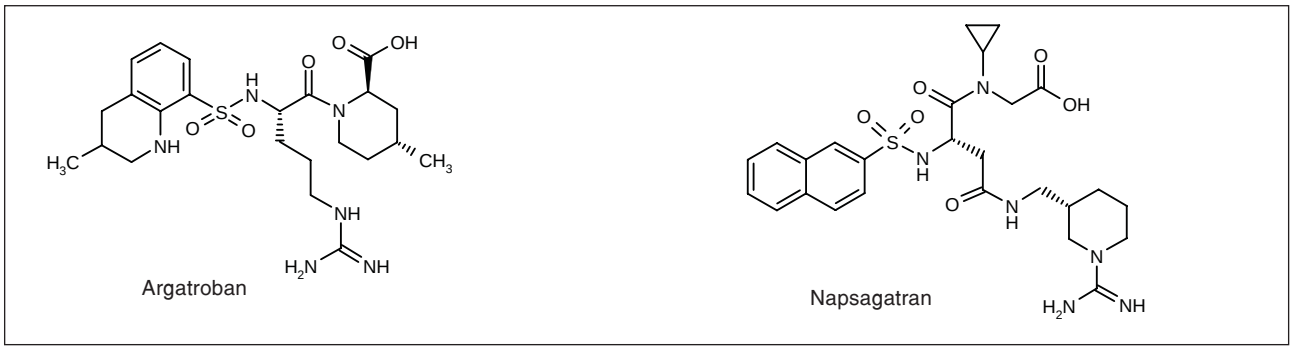

Fig. 3. Chemical structures of argatroban and napsagatran.

argatroban and napsagatran (Fig. 3), showed good tolerability and moderate pharmacokinetics but no oral bioavailability (30).

Argatroban is less potent than DuP-714 (K_i = 20 nM) and exhibits a moderate prolongation of the activated partial thromboplastin time (aPTT) *in vitro* (doubling of aPTT at ED_{200} = 1.4 μM) (31). It showed antithrombotic efficacy in several animal models of arterial thrombosis (32, 33). Argatroban was well tolerated and has been studied in several clinical trials; however, it showed no superiority compared to heparin as an adjunct to streptokinase in patients with acute myocardial infarction (6). On the other hand, it has been approved as an alternative to heparin in patients with heparin-induced thrombocytopenia. The results of clinical trials with argatroban in several indications have recently been reviewed (34).

Napsagatran is another example of guanidine-based inhibitors, which was discovered by rational drug design (35). It is characterized by very potent and selective enzyme inhibition, a short plasma half-life and low bioavailability, which favored its development as an antithrombotic agent for i.v. administration. In patients with deep vein thrombosis, napsagatran showed efficacy and safety comparable to heparin (36). However, clinical development was terminated in 1999.

A general feature of the synthetic thrombin inhibitors described above is the strongly basic guanidino group (pKa ~ 12-13), which greatly contributes to the enzyme affinity forming a salt bridge with Asp189. Due to the cationic or zwitterionic nature, these inhibitors show poor mucous membrane permeation. In addition, because of the short biological half-life and extensive hepatic first-pass effect, none of these first-generation inhibitors was useful for oral therapy.

Extensive structural modifications were undertaken in order to overcome these disadvantages. Replacement of the (cyclo)alkyl guanidine by benzamidine resulted in a slight reduction of the basicity (pKa ~ 11). By introduction of metabolically stable heterocyclic core structures, inhibitors with improved pharmacokinetics were discovered. These benzamidine inhibitors showed potent enzyme inhibition and strong antithrombotic efficacy *in vitro* and *in vivo* but suffered from limited bioavailability. Therefore, promising examples were transformed into prodrugs, which, in a few cases, showed oral activity in preclinical and clinical studies.

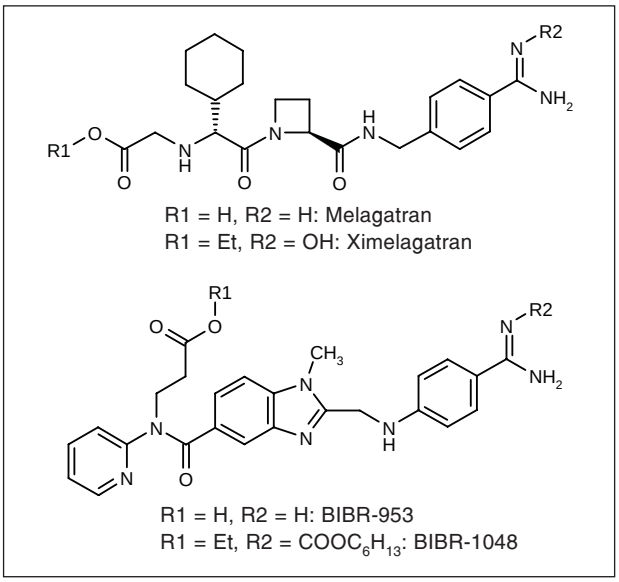

Fig. 4. Orally available thrombin inhibitors: melagatran, BIBR-953 and their respective prodrugs.

The most advanced compound in the field of orally active thrombin inhibitors is AstraZeneca's melagatran and its double prodrug ximelagatran (Fig. 4). The parent compound is a potent and reversible inhibitor (K_i = 2 nM) showing good selectivity *versus* related serine proteases except trypsin, which also is inhibited in the low nanomolar range. However, neither any side effects nor toxicity in animals related to the potent trypsin inhibition have been reported. Melagatran effectively prolongs coagulation parameters *in vitro* (*e.g.* aPTT, ED_{200} = 0.59 μM), indicating a high antithrombotic efficacy. Due to the three polar groups amidine, carboxylate and secondary amino group, which are mainly charged at pH 7, melagatran is highly water soluble (log K_D = –1.4) (37). The potential for penetrating the gastrointestinal barrier in the CaCo-2 cell permeability assay was shown to be rather low. Accordingly, the oral bioavailability of melagatran in healthy volunteer is only in the range of 5%.

The prodrug ximelagatran contains an ethyl ester group instead of the carboxylate and a weakly basic hydroxyamidine moiety, which is uncharged at pH 7. Ximelagatran is a rather lipophilic compound (log K_D = 0.9), which has a 170-fold higher preference for the lipid environment compared to melagatran. In addition, its CaCo-2 permeability is 80 times higher. After oral administration, reduction of the hydroxyamidine and cleavage of the ethyl ester occurs, resulting in the rapid formation of the parent compound with low interindividual variability in animal studies and in man.

In several clinical studies the combination of melagatran (s.c.) and ximelagatran (p.o.) or the use of the prodrug alone turned out to be as safe and effective as heparin or warfarin in the prevention and treatment of deep venous thrombosis (DVT). The efficacy of long-term ximelagatran treatment has been evaluated in comparison to warfarin as a preventive therapy for stroke in patients with arterial fibrillation. Ximelagatran was safe and effective, and the number of adverse events (stroke or major bleedings) was lower compared to warfarin. The results of preclinical and clinical studies with melagatran and ximelagatran have recently been reviewed (38).

A second benzamidine derivative under clinical development as double prodrug is BIBR-1048 (Fig. 4). After absorption and cleavage of the hexyl carbamate and ethyl ester moieties, BIBR-953 (Fig. 4) is released (39). The parent compound blocks thrombin activity in the lower nanomolar range (K_i = 4.5 nM). Because of its high polarity (log P: –2.4, octanol/buffer, pH 7.4) and low protein binding, BIBR-953 exhibits remarkable antithrombotic efficacy *in vitro* (aPTT, ED_{200} = 0.23 μM) (40). After i.v. administration BIBR-953 showed a half-life of 1.7 h in rats and 6 h in rhesus monkeys. The antithrombotic efficacy of BIBR-953 (i.v.) and BIBR-1048 (p.o.) was demonstrated in rat and rabbit models of venous thrombosis (41, 42). BIBR-953 was 4 times more potent than melagatran in reducing the clot weight without significant increase in bleeding tendency. In phase I clinical trials, oral administration of the prodrug BIBR-1048 showed a linear relationship between plasma concentration and coagulation parameters and a terminal half-life of the parent compound of 8-10 h (43, 44). BIBR-1048 is currently undergoing phase II clinical studies.

In order to improve oral bioavailability extensive research activities were focussed on the replacement of the permanently charged amidino or guanidino groups, which greatly hinder membrane penetration by less basic substitutes. Pioneer work in this field was done at Merck & Co., where low basic moieties such as aminopyridine have been discovered as substitutes for benzamidine or guanidine. Lead optimization culminated in the discovery of L-375378 (Fig. 5), a subnanomolar inhibitor (K_i = 0.5 nM, aPTT: ED_{200} = 0.4 μM) which showed excellent pharmacokinetics and bioavailability in different animal studies (dogs: 91%, rats: 42%, rhesus monkeys: 60%) (45). In addition L-375378 was fully efficacious in a rat model of arterial thrombosis *in vivo*. It was selected for clinical evaluation in 1998; however, results from clinical studies have not been presented so far.

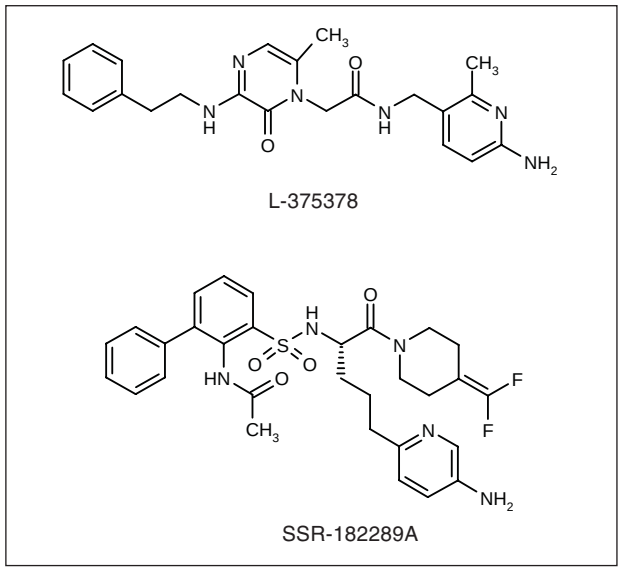

Fig. 5. Chemical structures of weakly basic thrombin inhibitors: L-375378 and SSR-182289A.

SSR-182289A (Fig. 5), a 3-aminopyridine derivative structurally related to argatroban, has recently been disclosed (46, 47). Despite its moderate potency *in vitro* (K_i = 31 nM, aPTT: ED_{200} = 0.8 μM) SSR-182289A showed antithrombotic efficacy after oral administration in several animal models comparable to ximelagatran.

Generally, the search for orally active thrombin inhibitors, bearing weakly basic or neutral moieties able to interact with the S1-subsite of the enzyme, turned out to be very difficult. On the one hand, potent enzyme inhibition *in vitro* and high oral bioavailability *in vivo* have been achieved. On the other hand, these types of inhibitors are generally more lipophilic and show higher unspecific protein binding, which results in a reduced antithrombotic efficacy *in vitro* and *in vivo* (48). Therefore, the biological and physicochemical properties have to be optimized simultaneously in order to obtain orally active, non-prodrug thrombin inhibitors with high antithrombotic efficacy.

Factor Xa

Factor X (fX) is a vitamin K-dependent glycoprotein with a MW of 59,000, synthesized in the liver. Posttranslational modification yields 11 gamma-carboxylic acid (gla) residues located within the amino-terminal light chain, which are responsible for the Ca^{2+}-dependent binding to negatively charged surfaces. fX circulates in the plasma as a disulfide linked heavy and light chain at a concentration of approx. 0.13 μM. The catalytic domain containing the serine protease catalytic triad is located near the carboxy-terminus and is activated by either the intrinsic factor tenase complex (fVIIIa, fIXa,

negatively charged phospholipids and Ca^{2+}) or the extrinsic tenase complex (fVIIa, TF, cellular surface and Ca^{2+}). fXa then serves as the physiologically most important enzyme component of the prothrombinase complex, which generates active thrombin from its precursor prothrombin. The critical position of fXa at the central convergence site of the extrinsic and intrinsic coagulation cascade, responsible for the generation of thrombin, makes it an ideal target for the pharmacological interruption of thrombin generation regardless of the initiating pathway. In addition, apart from its central role in the coagulation cascade, it has also been demonstrated that fXa stimulates TF expression and is able to trigger, *e.g.*, mitogenic as well as proinflammatory processes via its action on protease activated receptors (3). Preclinical studies also suggested that fXa inhibitors have a lower potential for bleeding complications than heparin, LMWH or thrombin inhibitors with similar (or even better) antithrombotic efficacy (49). The most favored explanation for this phenomenon is that reversible and competitive fXa inhibitors might not completely suppress the production of thrombin, which has a 10,000-fold higher affinity for platelet activation than for fibrinogen cleavage. Minimum amounts of thrombin might thus be sufficient to activate platelets and to induce normal hemostasis in the absence of significant thrombus formation.

Being a vitamin K-dependent enzyme like thrombin, fXa is inhibited by the oral anticoagulants such as warfarin or phenprocoumon. UFH and LMWHs also inhibit fXa in an AT-III-dependent manner. Whereas UFH exhibits an anti-Xa/anti-IIa-ratio of about 1, the LMWHs show a preference for fXa with anti-Xa/anti-IIa-ratios ranging from about 1.5-6.3, which results from different manufacturing processes for each individual LMWH. The pros and cons of these anticoagulants currently used in the clinical setting have already been discussed (7, 8, 10).

Considerable research efforts have focused on the identification of the minimum oligosaccharide sequence within the heparin molecule responsible for specific binding to AT-III. Scientists at Organon BV and Sanofi/Synthélabo were remarkably successful in developing the new sulfated pentasaccharide SR-90107A/Org-31540, which was obtained by total chemical synthesis. In contrast to UFH and LMWHs, this molecule has clearly defined physicochemical and pharmacological properties. SR-90107A/Org-31540, now called fondaparinux, is a selective inhibitor of fXa and has demonstrated remarkable antithrombotic efficacy in preclinical as well as clinical studies. The compound is absorbed rapidly and completely after subcutaneous administration, reaching half-maximum plasma levels after only 25 min. The elimination half-life is approx. 17 h and thus 4-5 times longer than those of LMWHs, making it suitable for once-daily administration. Fondaparinux does not need monitoring and dosage adjustment and has been recently shown to be superior to enoxaparin in several pivotal phase III clinical trials for the prevention of DVT in patients undergoing hip or knee surgery. Several excellent reviews concerning the preclinical and clinical profile of fondaparinux have been published recently (50-52). Fondaparinux (Arixtra™) was launched in the U.S. and in Europe in 2002.

A follow-up compound (SanOrg-34006, idraparinux) with an even higher affinity to human AT-III than fondaparinux (K_d =1.4 *vs.* 48 nM) and exceptionally long half-life in different species has also been published (53). Idraparinux is currently undergoing phase III clinical trials for the prevention of DVT in patients with orthopedic surgery. The published data suggest that idraparinux may be suitable for once-weekly s.c. administration.

In addition to other anticoagulant, antiaggregatory or fibrinolytic agents derived from leeches or insects, several direct, highly selective and potent peptidic inhibitors of fXa have been discovered and further characterized (54). Two proteins of this class, antistasin and tick anticoagulant peptide (TAP), have drawn much attention. Antistasin, a 119-amino acid protein, was isolated from the salivary glands of the Mexican leech, *Haementeria officinalis*, and exhibited potent and selective inhibition of fXa with a K_i in the low pM range (55).

Tick anticoagulant peptide (TAP), a 60-amino acid polypeptide, was characterized using extracts of the tick *Ornithodoros moubata* (56). TAP demonstrated reversible, slow, tight-binding inhibition of fXa with a remarkable K_d of 1.8 pM. Using recombinant technologies, both proteins became available in larger quantities and, due to their high potency and selectivity for fXa, have been extensively used as excellent research tools in order to elucidate the role of selective fXa inhibitors as antithrombotic agents (57). The antithrombotic efficacy of these inhibitors was comparable to other anticoagulants like hirudin and heparin and, in the case of recombinant TAP (rTAP), was achieved at levels which did not significantly increase bleeding parameters. However, despite these encouraging preclinical results, clinical development of these natural fXa inhibitors has not been pursued, at least in the case of antistasin due to its strong immunogenicity.

Similar to thrombin, the molecular structure of fXa has been resolved by X-ray crystallography and the active site of the enzyme has been described in detail (58, 59). These results formed the basis for rational drug design of synthetic, small-molecule fXa inhibitors, which are independent of AT-III and thus have the advantage of inhibiting free fXa as well as fXa bound to the prothrombinase complex. Therefore, synthetic fXa inhibitors may offer superior antithrombotic efficacy compared to the AT-III-dependent coagulation inhibitors.

DX-9065a (Fig. 6) is the prototype of a selective, synthetic fXa inhibitor derived from bisamidine precursors of moderate potency (60). It was shown to interact directly with the active site of the enzyme (61) and exhibits a K_i for human fXa of 41 nM (62). However, it should be mentioned that species differences exist concerning the inhibitory effect against fXa with human fXa being the most sensitive (63). Nevertheless, the compound showed dose-dependent anticoagulant and antithrombotic effects in animal models of thrombosis (64) and septicemia (65).

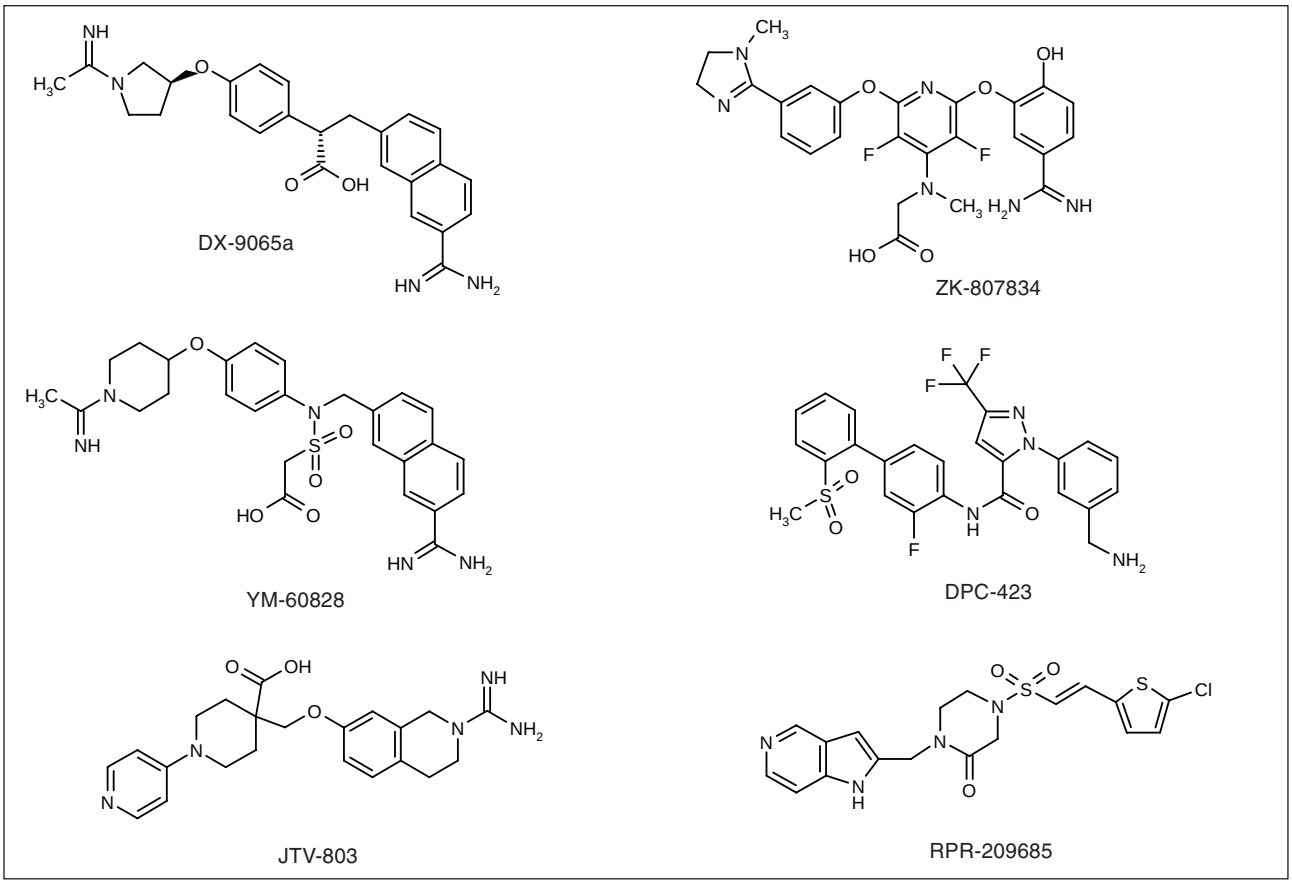

Fig. 6. Chemical structures of low-molecular-weight factor Xa inhibitors.

In comparison to heparin, LMWH or argatroban, which prolonged bleeding time at a dose slightly higher than the effective dose, DX-9065a did not increase the bleeding time, suggesting a favorable benefit/risk ratio (66). DX-9065a was also shown to be a potent inhibitor of free as well as prothrombinase-bound fXa (67). Recent data also revealed that DX-9065a inhibits fXa-induced tissue factor expression in human monocytes and endotoxemic rats (68) and modulates leukocyte-endothelial cell interaction in endotoxin-treated rats (69), suggesting that fXa may be an important modulatory factor in septicemia. Although antithrombotic efficacy after oral administration of DX-9065a has been reported in different species, bioavailability appears to be rather low and the compound has been advanced with an injectable formulation into clinical phase I and II trials with encouraging results reported recently (70, 71). DX-9065a is currently in phase II trials for the prevention of MI in patients with unstable angina pectoris.

ZK-807834, another bisamidine derivative (Fig. 6), inhibits fXa with a K_i of 110 pM (72) showing a >2000-fold selectivity against homologous serine proteases such as trypsin. In human *in vitro* coagulation assays, ZK-807834 prolonged prothrombin time (PT) and aPTT in the range of 0.3-0.5 μM and inhibited fXa in the clot bound prothrombinase complex *in vitro* with an IC_{50} of 10 nM (73). In a rabbit model of arterial and venous thrombosis, ZK-807834 showed antithrombotic efficacy at lower doses and with less effects on hemostasis compared to rTAP and DX-9065a (74). ZK-807834 demonstrated dose-dependent antithrombotic effects in a canine thrombosis model with only minimal changes in *ex vivo* coagulation parameters and generally less bleeding compared to equieffective doses of enoxaparin (75). It also decreased early reocclusion and improved 24-h patency more effectively than either rTAP, heparin or aspirin in a canine model of fibrinolysis after acute thrombotic occlusion of the coronary artery (76). In a rabbit venous shunt model of thrombosis, ZK-807834 also very effectively inhibited thrombus formation with a favorable efficacy-to-bleeding ratio (77). As also observed with DX-9065a, it was demonstrated in a recent publication that ZK-807834 blocked fXa-mediated cellular responses, which are potentially involved in cell proliferation and local inflammation (78). ZK-807834 has shown oral bioavailability in several animal species including primates (79) and has been advanced to clinical development. The compound has been reported to be in phase II clinical trials for the potential parenteral treatment of unstable angina pectoris.

Based on DX-9065a, the bisamidine derivative YM-60828 (Fig. 6) was discovered, which showed about 20-fold higher *in vitro* potency (K_i = 2.3 nM) (80). The compound showed oral bioavailability in the range of 20% (squirrel monkey) and 33% (guinea pig) and antithrombotic effects in different *in vivo* models (80). YM-75466, the methanesulfonate salt of YM-60828, is currently undergoing clinical development, and potent successor compounds with improved oral bioavailability have also been described recently (81).

In contrast to the fXa inhibitors discussed before DPC-423 (Fig. 6) contains a benzylamino moiety instead of a highly basic amidine group interacting with Asp-189 of the enzyme. DPC-423 has been described recently as a potent and orally available nonpeptide fXa inhibitor (82). It inhibits fXa with a K_i of 0.15 nM with good selectivity against other components of the coagulation and fibrinolytic system. In accordance with its favorable physicochemical properties, DPC-423 showed high CaCo2-permeability and exhibited excellent bioavailability in dogs (57%) and rats (36%) which was associated with a low clearance in both species. The compound was very effective in rat and rabbit models of arteriovenous shunt thrombosis (83). Interestingly, in a rabbit arterial thrombosis model, the combination of aspirin and DPC-423 at otherwise ineffective doses produced a significant antithrombotic effect, suggesting that this combination might be useful for the prevention of arterial thrombosis (84). First clinical results showed that the compound is well tolerated and orally available with a plasma half-life in the range of 27-35 h, which seems sufficient for once-daily dosing. Recently, new nonbenzamidine follow-up fXa inhibitors have been disclosed which show an even higher bioavailability of 74% in dogs (85).

Several fXa inhibitors are currently in clinical development by Japan Tobacco (JTV-803), Aventis (HMR-2906), Teijin (TC-10), Cor Therapeutics (MLN-1021) and Kissei (KFA-1982). However, with the exception of JTV-803 (Fig. 6), no structure has been disclosed thus far for any of the other compounds. Many companies still have active research programs in the field of fXa inhibitors, and significant progress has been reported during the last months concerning potency, selectivity and, most impressive, oral bioavailability. These compounds are exemplified by RPR-209685 (Fig. 6), which showed a K_i of 1.1 nM and an excellent oral bioavailability of 97% in dogs (86).

Based on the enormous progress during the last years in the development of orally active fXa inhibitors useful for clinical evaluation, this class of compounds is expected to play an important role in the treatment and prophylaxis of thrombotic disorders in the future.

Factor VIIa

Factor VIIa (fVIIa) plays a key role in the initiation of the extrinsic coagulation cascade. The related zymogen, factor VII (fVII), is a single chain, vitamin K-dependent glycoprotein, which is present in blood in a concentration of about 10 nM. Approximately 1-2% of circulating fVII are in the activated form but exhibit low enzymatic activity, because the active site is not efficiently expressed (2). Upon damage of the endothelial layer or the blood vessel wall, circulating fVII rapidly associates with its cofactor, the cell surface receptor tissue factor (TF) in a Ca^{2+}-dependent manner. The TF/fVIIa complex increases the catalytic activity of fVIIa towards its physiological substrates, fIX and fX, by about 5500-fold (87). In the presence of cell surface and Ca^{2+}, the fVIIa/TF complex effectively converts fX into fXa, which in turn generates a small amount of thrombin. Thrombin and fXa activate the procofactors fV and fVIII. fIXa and fVIIIa form the intrinsic tenase complex, an efficient catalyst for the activation of fX. fXa and activated fV are the major components of the prothrombinase complex, which efficiently converts prothrombin into thrombin.

The biological activity of fVIIa is controlled by tissue factor pathway inhibitor (TFPI), a protein that inhibits the TF-fVIIa complex in a fXa-dependent manner. In the presence of fXa the inhibitory potency of TFPI *versus* fVIIa/TF complex is 50-fold higher, resulting in a feedback inhibition of fVIIa/TF (88). About 85% of endogenous TFPI is located in the microvascular endothelium, from which its release is induced by heparin (89). Recombinant TFPI (tifacogin) is under clinical development as a potential treatment of sepsis. In a phase II study with 210 patients suffering from severe sepsis, a trend toward a reduction in 28-day all-cause mortality was observed (90). However, in 2001 the phase III trial “Optimist” did not reach the primary efficacy endpoint and currently the development status of tifacogin is unknown.

Nematode anticoagulant protein c2 (NAPc2) is an 84-amino acid protein isolated from the hookworm *Ancyclostoma canium*, which, similarly to TFPI, inhibits fVIIa/TF after binding to its cofactor fX/fXa. In contrast to TFPI, which interacts with the fXa active site prior to fVIIa/TF inhibition, NAPc2 and the 85-amino acid recombinant form rNAPc2 bind to an exosite of either fX or fXa (91). This binary complex inhibits fVIIa/TF with an inhibition constant of about 10 pM. In canine models of arterial and venous thrombosis the structurally related rNAP5 showed antithrombotic efficacy (92). In addition, rNAPc2 effectively blocked the fVIIa/TF-driven thrombin generation in a monkey model of endotoxin-induced sepsis (93). The therapeutic potential of rNAPc2 was studied in a number of clinical trials. In patients undergoing total knee replacement it was found to be 50% more effective than heparin in the prevention of DVT (94). In addition, it is also under clinical evaluation in patients with unstable angina (phase II) and disseminated intravascular coagulation (phase I).

An alternative approach using the TF/fVIIa complex as a target for antithrombotic therapy is recombinant fVIIa, in which the active site is irreversibly blocked by reaction with Phe-Phe-Arg-chloromethyl ketone (FFR-rFVIIa or fVIIai). This protein retains its TF binding capacity but is enzymatically inactive. fVIIai competes with native fVIIa for TF binding, which results in potent antithrombotic activity *ex vivo* and *in vivo* (95, 96).

Similarly, a modified, soluble tissue factor mutant (sTF or hTFAA) has been developed, which binds fVIIa with kinetics and affinity equivalent to wild-type TF. In contrast, the hTFAA/fVIIa complex showed a 34-fold reduction in catalytic efficiency for fX activation (97). In preclinical studies hTFAA, as well as the rabbit homologue of this mutant, displayed antithrombotic efficacy in a rabbit model of arterial thrombosis comparable to heparin, but with lower bleeding tendency.

A series of allosteric peptide inhibitors of fVIIa have been discovered, from which the most potent examples E-76 (18 amino acids) and A-183 (15 amino acids) blocked the fX activation at low nanomolar concentration. As indicated by X-ray structures of fVIIa/inhibitor complexes, both peptides bind to different exosites of fVIIa (98, 99). A-183 potently prolonged clotting in the prothrombin assay but had no effect on the aPTT (100). Recently, fusion peptides of A-183 and E-76 have been discovered showing similar binding affinity to fVIIa but increased extent of inhibition of fX activation compared to the precursor peptides (101).

Increasing research efforts are currently ongoing in order to develop small-molecule inhibitors of TF/fVIIa. Although a number of patent application from E. Merck, Aventis, AstraZeneca, Hoffmann-LaRoche and other companies have been published in the last two years, only limited information about the biological properties of these new inhibitors is available to date. Ro-67-8698 (Fig. 7) is a potent fVIIa inhibitor (IC_{50} = 8 nM) which showed a moderate selectivity *versus* related serine proteases (thrombin: IC_{50} = 0.6 μM, fXa: IC_{50} = 0.4 μM, trypsin: IC_{50} = 2.2 μM). After s.c. administration of 30 mg/kg to guinea pigs, Ro-67-8698 caused a 2.5-fold PT prolongation but had only minor effects on bleeding parameters. In contrast, the selective thrombin inhibitor napsagatran at comparable PT prolongation exhibited a significantly higher bleeding tendency, suggesting that fVIIa inhibition might be a safer principle than thrombin inhibition (102).

The synthesis of the benzamidino-based inhibitor compound [I] (Fig. 7), originally discovered by Ono Pharmaceutical Company, was published recently (103). The reported enzyme inhibition in the low nanomolar range was confirmed (K_i = 6.4 nM), although no further information on the biological activity of the compound is available.

Starting from potent and selective thrombin inhibitors, dual thrombin/fVIIa inhibitors of moderate potency such as compound [II] (Fig. 7, thrombin: IC_{50} = 0.21 μM, fVIIa: IC_{50} = 0.41 μM) have been designed based on the X-ray structures of both enzymes (104).

As shown above, the low-molecular-weight inhibitors of fVIIa known so far belong to the class of benzamidines which generally suffer from low intestinal absorption and short half-life *in vivo*. Based on the considerable efforts spent on the development of orally active thrombin and fXa inhibitors, it can be expected that the discovery of potent, selective, orally active and clinically useful fVIIa inhibitors will be a similar challenge for medicinal chemistry.

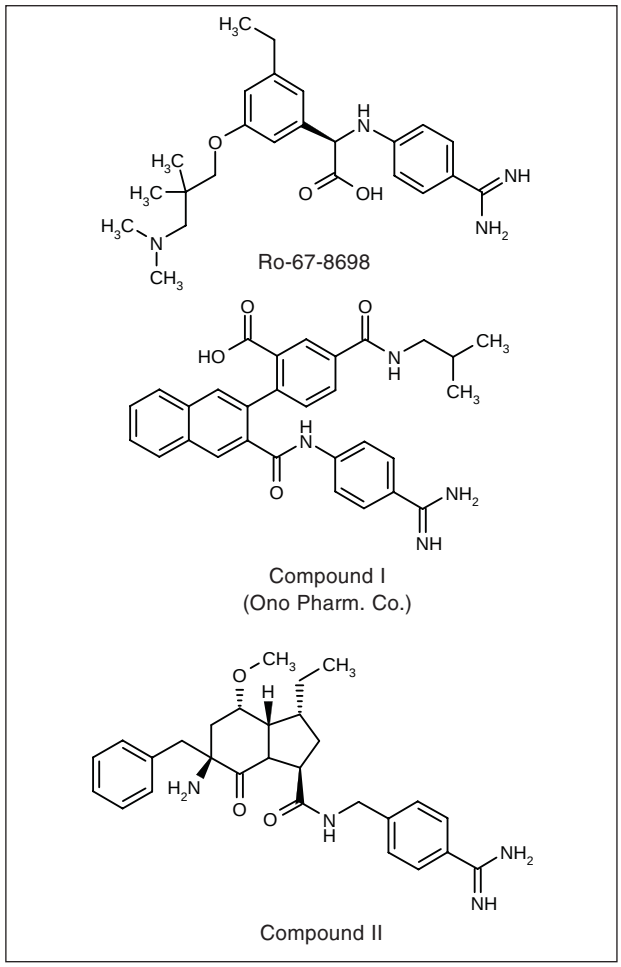

Fig. 7. Chemical structures of low-molecular-weight factor VIIa inhibitors.

Factor IXa

Coagulation factor IX (fIX) is a vitamin K-dependent serine protease with a molecular weight of 65 kDa. fIX plays a key role in the intrinsic pathway of blood coagulation and is activated either by TF/fVIIa or by fXIa in the presence of Ca^{2+}. Activated factor IX (fIXa) transforms fX in the presence of fVIIIa and Ca^{2+} ions on the surface of platelets or endothelial cells into the activated form fXa.

fIXa and fXa play distinct roles in the tissue factor-dependent initiation of coagulation (106). fXa seems to be responsible for the activation of platelets by generating small amounts of thrombin in the vicinity of platelets. fIXa generates fXa on the platelet surface leading to the subsequent formation of the prothombinase complex and thrombin generation. Consequently, fIXa is essential for amplification of coagulation, an observation highlighted by the bleeding tendency associated with fIX deficiency (hemophilia B). Accordingly, high fIX levels are associated with increased risk of venous thromboembolism and fIXa levels are increased in patients with acute coronary

artery thrombosis (106). Due to the increasing evidence of fIXa as a potential target for antithrombotic therapy, two different strategies have been developed thus far: active site-blocked fIXa and monoclonal antibodies against fIX/fIXa.

Active site-blocked fIXa (fIXai) is prepared by alkylation of the fIXa active site with Glu-Gly-Arg-chloromethylketone (107). This irreversibly blocked fIXa competes with endogenous fIXa for incorporation into the intrinsic tenase complex that assembles on the surface of activated platelets. In a canine model of coronary thrombosis, i.v. administration of fIXai dose-dependently prevented thrombus formation without affecting the hemostatic variables. In contrast, animals given heparin at a concentration sufficient to prevent occlusive thrombosis showed markedly increased bleeding. Similar results were obtained with fIXai in comparison to heparin in a canine model of cardiopulmonary bypass (108). In a guinea pig model of arterial thrombosis, fIXai at a dose of 60 μg/kg reduced the thrombosis index by about 90% without significant prolongation of PT, aPTT and bleeding time (109). Active site-blocked fXa and heparin, which were used in this study for comparison, also showed a dose-dependent inhibition of thrombus formation. Interestingly, at equiefficacious doses fIXai showed the lowest bleeding tendency, indicating a favorable therapeutic ratio.

Monoclonal antibodies are the alternative approach for blocking the activity of fIX/fIXa. The murine fIX monoclonal antibody BC2 has been generated and evaluated for its capacity to prolong aPTT and to prevent arterial thrombosis in the rat (110). BC2 demonstrated complete antithrombotic efficacy without effects on blood loss and only a limited prolongation of the aPTT. Heparin, at doses that reduced thrombus mass to a similar extent, prolonged aPTT over 500-fold and increased blood loss significantly. Similar results with BC2 were obtained in a rat model of venous thrombosis (111).

As described before, fIXa in complex with its cofactor fVIIIa assembles into the intrinsic tenase complex on the surface of activated platelets or endothelial cells and converts fX into fXa. Ca^{2+}-dependent cell surface binding of fIX/fIXa is mediated by the gamma-carboxyglutamic acid rich domain (Gla domain) (112, 113). The human antibody 10C12 specifically binds to the Gla domain of fIX and interferes with all known coagulation processes that involve fIX/fIXa (114). In rat and guinea pig models of arterial thrombosis, 10C12 turned out to be a potent antithrombotic agent without affecting normal hemostasis.

Similarly, the anti-fIXa monoclonal antibody SB-249417 showed a superior antithrombotic efficacy compared to the low-molecular-weight heparin enoxaparin in a rat model of arterial thrombosis (115). Recently, the protective properties of SB-249417 in a rat model of thromboembolic stroke have been described (116). Inhibition of fIXa within 4 h after thromboembolic stroke was compared with the effects of t-PA. Both SB-249417 and t-PA reduced infarct volumes by about 40%, but only the fIXa antibody also improved neurological deficits, whereas t-PA in this case had no effect.

In general, the results discussed above clearly indicate that selective inhibition of fIXa is an effective antithrombotic strategy. However, to date selective, low-molecular-weight inhibitors of fIXa have not been described.

Factor XIa

Factor XI (fXI) circulates in the plasma as a non-covalent complex with high-molecular-weight kininogen (HMKW) at a concentration of ~ 30 nM. fXI deficiency occurs in all racial groups, but is particularly common in Ashkenazi Jews (117). It is generally associated with relatively mild bleeding, although excessive bleeding has been observed with trauma or surgery. On the other hand, high levels of fXI have been demonstrated to be a risk factor for venous thrombosis (118). fXI is converted to its active form fXIa through enzymatic cleavage by fXIIa. However, it has also been shown that thrombin is capable of activating fXI on activated platelets (119). fXIa participates in the intrinsic coagulation pathway by activating fIX either as free fXIa or in complex with HMWK (120). Activation of fXI by thrombin also appears to be involved in the inhibition of fibrinolysis, and neutralization of fXI by a polyclonal antibody was shown to enhance thrombolysis in a rabbit jugular vein thrombosis model (121). Endogenously, the fXIa proteolytic activity is regulated by members of the serine protease inhibitor family, of which α_1-antitrypsin and, to a lesser extent, AT-III are the physiologically most important, followed by C1 inhibitor and α_2-antiplasmin. Recently, it has been shown that protease nexin-1 is also a potent inhibitor of fXIa (122). However, to date no small-molecule, synthetic inhibitors of fXI have been published in the literature.

Factor XIIa

Factor XII (fXII), also called Hageman factor, is a glycoprotein zymogen present in the plasma at a concentration of ~ 0.4 μM. fXII, prekallikrein and HMWK constitute the contact phase of intrinsic coagulation. When assembled on an electronegative surface these three proteins initiate the rapid generation of fXIIa (123). fXII deficiency is an inherited disorder that causes prolonged clotting (coagulation) of blood in a test tube without a clinical bleeding tendency. This suggests that other proteases within the coagulation cascade may compensate for intrinsic coagulation activation in the absence of fXIIa. Several fXIIa inhibitory antibodies have been described in the literature (124). However, the absence of clinical symptoms such as bleeding may be the reason why this serine protease, although very upstream in the coagulation cascade, has not been a preferred target for pharmacological interruption of the intrinsic coagulation pathway. Consequently, no synthetic inhibitors have been published so far.

Activated protein C

Protein C is a vitamin K-dependent zymogen with a MW of 62,000 and circulates in plasma at a concentration of ~0.06 μM as the precursor form of activated protein C (aPC). Activation of protein C occurs on the surface of endothelial cells, on which thrombin is bound to the receptor protein thrombomodulin. In contrast to the other vitamin K-dependent coagulation factors, aPC functions as an anticoagulant by catalyzing the proteolytic inactivation of fVa and fVIIIa. In addition, aPC also contributes to the fibrinolytic response by a complex formation with plasminogen activator inhibitor 1.

Two types of protein C deficiency have been described leading to an increased risk for thrombotic complications (125). Type I deficiency is characterised by a decrease in protein C activity and antigen levels caused by a low rate of synthesis or reduced stability of the molecule which otherwise functions normally. In type II deficiency, the decrease in protein C activity is greater than that of the antigen level due to synthesis of an abnormal protein C molecule. In addition, aPC resistance is an inherited disorder of fV and is associated with venous thromboembolism (126). This disorder is characterised by a low anticoagulant response of plasma upon addition of aPC. The abnormality has been reported in 20-60% of patients with venous thromboembolism and a single gene mutation (factor V Leiden) has been described in >90% of aPC-resistant patients.

Due to the physiological role of aPC, the strategy in drug development has been directed toward the support of or to supplement the endogenous anticoagulant and profibrinolytic effects of aPC under certain pathophysiological conditions. Of importance in this respect are the results from the recently published PROWESS study, where administration of recombinant human aPC to patients with severe sepsis demonstrated an impressive reduction of the relative risk of death by 19.4% compared to placebo (127).

Conclusions

The serine proteases and coagulation factors IIa (thrombin), VIIa, IXa, Xa, XIa and XIIa have gained different attention as targets for antithrombotic therapy. Indirect AT-III-dependent inhibitors of thrombin and fXa such as heparin or LMWH are established in the treatment and prophylaxis of thromboembolic diseases. In addition, the selective, indirect fXa inhibitor fondaparinux (pentasaccharide) showed superior clinical efficacy compared to enoxaparin and may play an important role in the future.

Low-molecular-weight direct inhibitors of thrombin and fXa have been developed in the last decade aiming at an oral antithrombotic treatment. Despite the enormous efforts to design orally active coagulation inhibitors with high antithrombotic efficacy, only a very limited number of compounds are under late clinical development so far. A promising approach for solving the problem of low bioavailability are the benzamidine prodrugs ximelagatran and BIBR-1048, which are the front-runners in the field of orally active thrombin inhibitors and have already demonstrated clinical efficacy in the setting of DVT prevention. On the other hand, weakly basic and orally active inhibitors of thrombin and fXa may constitute an alternative and promising concept. However, clinical efficacy of compounds like SSR-182289 and DPC-423 remains to be demonstrated.

fVIIa and fIXa, although located more upstream within the extrinsic and intrinsic part of the coagulation cascade, have gained less attention as pharmacological targets than thrombin and fXa so far. Here biological intervention is mainly based on inhibitory peptides (*e.g.*, NAPc2 as fVIIa inhibitor), active site-blocked enzymes (fVIIai and fIXai) or, in the case of fIXa, monoclonal antibodies. However, in the last two years an increasing amount of scientific reports and patent applications concerning synthetic, low-molecular-weight fVIIa inhibitors has emerged. With the exception of NAPc2, which is under clinical development in late stages and for different indications, inhibitors of fVIIa and fIXa have been studied predominantly in preclinical studies. Despite the promising results reported so far, it has to be clarified whether this will translate into higher clinical efficacy or improved safety.

fXIa and fXIIa, responsible for the initiation of the intrinsic coagulation pathway, only play a minor role in thrombosis research. Although high plasma levels of fXI seem to be a risk factor for venous thrombosis and macromolecular inhibitors of fXIa have recently been described, it is unclear whether fXIa will be a useful target for pharmacological intervention in the future. Similarly, the data from the literature suggest that inhibition of fXIIa may not be an attractive target for effective antithrombotic therapy.

Successful enhancement of the natural endogenous anticoagulant and profibrinolytic pathways by the use of recombinant human aPC resulted in a reduction in mortality in patients with severe sepsis. Thus, aPC may possibly represent a new cornerstone in the treatment of septicemia.

Given the currently available preclinical and clinical data on pharmacological inhibition of these serine proteases, it can be envisaged that within a few years from now the low-molecular-weight direct inhibitors of thrombin and fXa will constitute a modern long-term oral therapy of thromboembolic diseases. Given the obvious potential advantage of lacking the drawbacks of the currently used oral anticoagulants, these agents may offer a substantial therapeutic progress and may add considerable value to the already existing armamentarium of antithrombotic agents.

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