Drugs of the Future 1998, 23(4): 423-436

CCC: 0377-8282/98

Review Article

Thrombin and factor Xa inhibitors

Brigitte Kaiser

Center for Vascular Biology and Medicine Erfurt, Unit of Experimental Angiology, Friedrich Schiller University Jena, Nordhäuser Str. 78, D-99089 Erfurt, Germany

CONTENTS

Summary

The serine proteases thrombin and factor Xa are key enzymes in the cascade-like activation of the coagulation system and, therefore, they are at present the most important targets for the development of new anticoagulant/antithrombotic drugs which may be advantageous over heparin and heparin-related agents for prophylaxis and/or treatment of thromboembolic disorders. Directly acting thrombin and factor Xa inhibitors are either derived from natural sources, such as hirudin as antithrombin or antistasin and tick anticoagulant peptide as anti-factor Xa agents, or they are chemically synthesized such as the thrombin inhibitor argatroban or the factor Xa inhibitor DX-9065a. Due to the inactivation of thrombin or factor Xa, anticoagulant effects are obtained which can be measured in global clotting assays. The inhibition of thrombin by highly effective and selective compounds prevents clotting of blood but does not effectively block the further generation of thrombin. In contrast, the inactivation of factor Xa results in a strong reduction of thrombin generation. Both direct thrombin and factor Xa inhibitors are able to inhibit clot-associated thrombin and factor Xa, which play important roles in the thrombus-associated procoagulant activity and the continuation of thrombotic processes *in vivo*.

Comprehensive experimental studies suggest that thrombin and factor Xa inhibitors may be effective drugs in a wide range of intravascular thrombus formation. The inactivation of thrombin and factor Xa, which are both known to be potent mitogens, may also have potential implications for the inhibition of vascular restenosis. Recent clinical trials revealed the usefulness of direct thrombin inhibitors in various thrombotic and cardiovascular indications, but also an increased risk of bleeding complications. It is assumed that factor Xa inhibitors prevent thrombus formation without compromising hemostasis.

In conclusion, both thrombin and factor Xa inhibitors are promising drugs for the management of various thrombotic disorders which has to be further demonstrated under clinical conditions.

Introduction

Thromboembolic disorders are a major cause of morbidity and mortality in industrialyzed countries. Whereas a balanced activity of the clotting system is necessary to prevent blood loss after vessel injury, an uncontrolled activation of the coagulation system within the blood vessel can cause various cardiovascular disorders, and, thus, represents a serious medical problem which requires an effective prophylaxis and/or treatment by anticoagulant/ antithrombotic agents.

At present, the most important targets for the development of new anticoagulants/antithrombotics are thrombin and factor Xa. Thrombin is a trypsin-like serine protease which is directly generated from its vitamin K-dependent precursor protein prothrombin at the end of a cascade-like series of events by splitting off the F1+2 prothrombin fragment (1, 2). The initiation of coagulation primarily includes the exposure of tissue factor and its association with factor VIIa resulting in the activation of both factor IX and factor X (Fig. 1). The activated clotting factors are essential for the induction of thrombin generation and the thrombin-mediated reactions that follow. Thrombin, which exerts both procoagulant and anticoagulant effects, plays a key role in the regulation of hemostasis and thrombosis. It stimulates platelet activation, catalyzes the conversion of fibrinogen into clottable fibrin by releasing fibrinopeptides A and B, activates factor XIII to cross-link soluble fibrin to form the final clot and amplifies its generation by converting the cofactors V and VIII into their active forms. On the contrary, thrombin binds to thrombomodulin on endothelial cells, and thus it activates the protein C pathway. Due to the resulting inactivation of

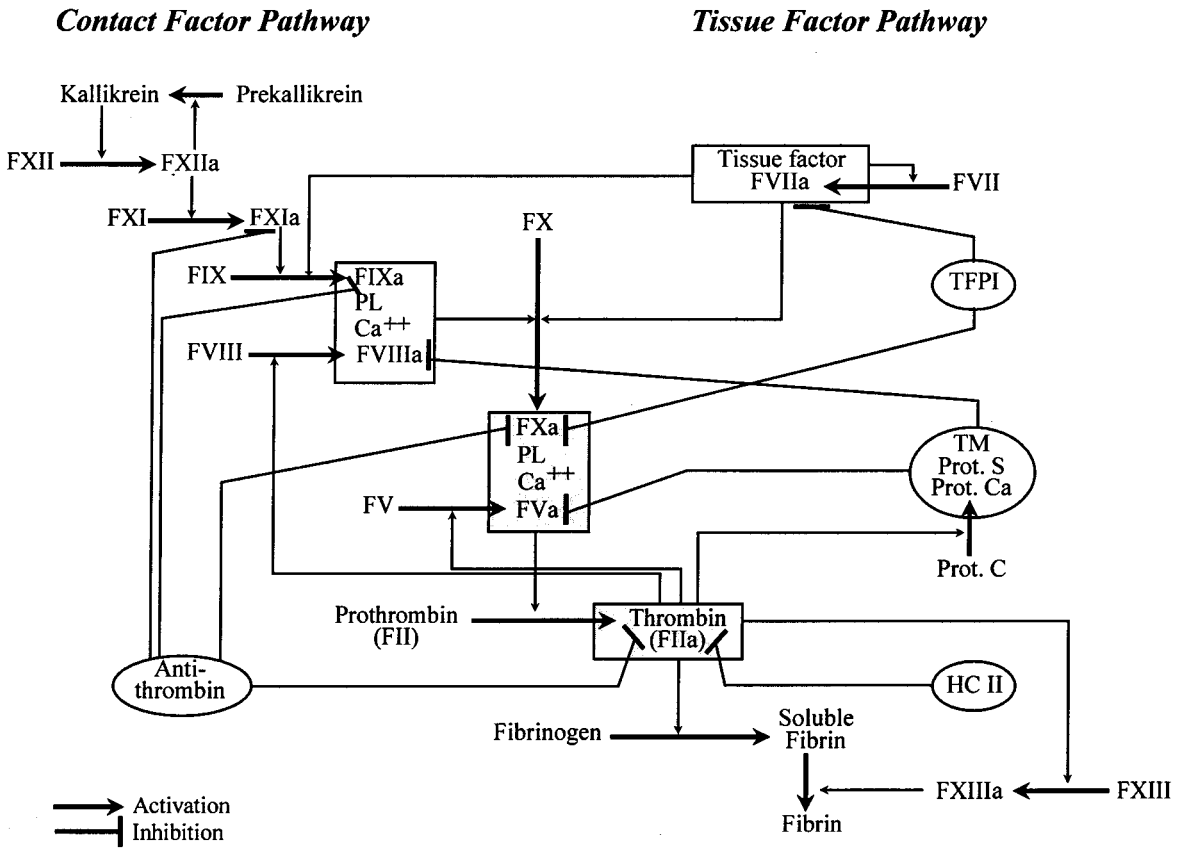

Fig. 1. Scheme of contact and tissue factor-induced activation of the plasmatic coagulation system.

factors Va and VIIIa, it inhibits its own generation. Thrombin is a multifunctional protein which has, besides its role in blood coagulation, numerous cellular effects, *e.g.*, on endothelial cells, smooth muscle cells, fibroblasts and macrophages (3, 4). Activation of platelets as well as the majority of thrombin-stimulated cell responses are mediated by the binding of the enzyme to its specific cell surface receptor which belongs to the seven transmembrane domain receptor family coupled to heterotrimeric guanine nucleotide-binding proteins (G-proteins). After binding to and subsequent cleavage of the extracellular domain of the receptor by thrombin, a new amino terminus is formed which acts as a "tethered" ligand activating the receptor and the resulting intracellular signal transduction (5).

The conversion of prothrombin to thrombin is catalyzed by the prothrombinase complex which is assembled on phospholipid surfaces and involves the Ca^{2+}-dependent association of factor Xa and factor Va. Membrane surfaces required for prothrombinase complex formation are provided after vascular cell damage and activation of platelets. Factor X is synthesized in the liver as vitamin K-dependent protein and secreted into the blood stream as an inactive precursor. It circulates in plasma as a two-polypeptide chain glycoprotein with a molecular weight of 58,000 Da. Factor Xa is generated either by factor IXa which converts factor X into its active form in the presence of factor VIIIa, Ca^{2+} and phospholipids or by the tissue factor/factor VIIa complex (Fig. 1). Activated platelets, endothelial cells at sites of vascular injury, vascular smooth muscle cells (VSMC) and fibroblasts are capable of supporting the activation of factor X into factor Xa (6). Like thrombin, factor Xa is also a very important enzyme in the coagulation cascade especially because of its central position at the stage of the common pathway after contact- or tissue factor-induced coagulation. Compared to thrombin, factor Xa acts at an earlier level in the coagulation system and is not such a multifunctional protein, *i.e.*, it exerts its action mainly on the substrate prothrombin. Whereas thrombin effectively cleaves its substrate fibrinogen which is dissolved in plasma, the catalytic activity of factor Xa alone is extremely low and is insufficient to generate thrombin in amounts that are able to produce a fibrin/platelet clot at the site of vascular injury or inside the vessel (7, 8). However, upon formation of the prothrombinase complex, the catalytic efficiency of thrombin synthesis by factor Xa is enhanced by approximately 300,000-fold so that sufficient amounts of thrombin can be generated. Vascular endothelial cells, smooth muscle cells and platelets are known to express

effector cell protease receptor (EPR-1), a 65-kDa membrane receptor for factor Xa, which may be of importance in the regulation of prothrombinase-catalyzed thrombin generation, as well as in the mediation of vascular cell signalling following receptor occupancy by locally generated factor Xa (9-11).

The activity of clotting enzymes, including thrombin and factor Xa, is controlled by several endogenous inhibitors. Antithrombin III (AT III) is a potent inhibitor of the blood coagulation system that, by interaction with glycosaminoglycans, effectively inactivates both thrombin and factor Xa, as well as other serine proteases (12). However, factor Xa which is bound to factor Va on the membrane surface does not seem to be susceptible to AT III-mediated inhibition (7, 13). Heparin cofactor II (HC II) also inactivates thrombin in a reaction that is catalyzed by dermatan sulfate (14). Protein C, which is activated (APC) by the thrombin-thrombomodulin complex, inactivates the cofactors Va and VIIIa in the presence of the cofactor protein S (15). After cleavage by APC, the factor Va molecule is incapable of binding factor Xa and prothrombin, which results in an inhibition of thrombin generation. The extrinsic coagulation pathway is controlled by the tissue factor pathway inhibitor (TFPI). In a first step, TFPI binds and inhibits factor Xa, and in a second step, the TFPI-factor Xa complex inhibits the tissue factor-factor VIIa complex (16).

Because of the central regulatory roles of thrombin and factor Xa in the coagulation cascade, it is necessary to analyze whether the direct inhibition of thrombin and/or factor Xa by various substances (Fig. 2) is a suitable way to interfere with the activation of blood coagulation and which inhibitors are more competent for use as anticoagulants/antithrombotics in clinical states.

Thrombin inhibitors

Because of the central role of thrombin in the coagulation cascade, the enzyme was the primary target for the development of anticoagulant/antithrombotic drugs, and most currently used anticoagulant regimens are based on inhibition of the activity of thrombin. With respect to its natural substrates, thrombin cleaves peptide bonds of arginine which is fixed in the specificity pocket of the enzyme via its guanidinoalkyl side chain (17). Secondary binding sites in the vicinity of this pocket can interact with the leaving group of the substrate and with further groups so that the cleavable bond comes close to the active center serine hydroxyl. Blocking of the active site of the enzyme by a fast reaction with an inhibitor leads to the decrease or disappearance of thrombins enzymatic activity.

The prototype of directly acting antithrombins is hirudin. Hirudin which is originally produced in salivary glands of the medicinal leech, *Hirudo medicinalis,* is the most potent and selective thrombin inhibitor known. It is a 7 kDa, single-chain polypeptide which contains 65 amino acids; the recombinant form, produced by *E. coli* or in yeast, is a desulfatohirudin lacking the sulfate residue on tyrosine 63. Hirudin forms a noncovalent complex with thrombin utilizing the anion-binding exosite as well as the apolar binding site adjacent to the active site of thrombin. It blocks sterically the access of both natural and synthetic substrates to the active site of the enzyme (18). Coupling of recombinant hirudin to polyethylene glycol (PEG-hirudin) leads to longer-lasting agents with an increase in the efficacy of the drug (19). A new, naturally occurring potent thrombin inhibitor is triabin, isolated from the saliva of the assassin bug *Triatoma pallidipennis*,

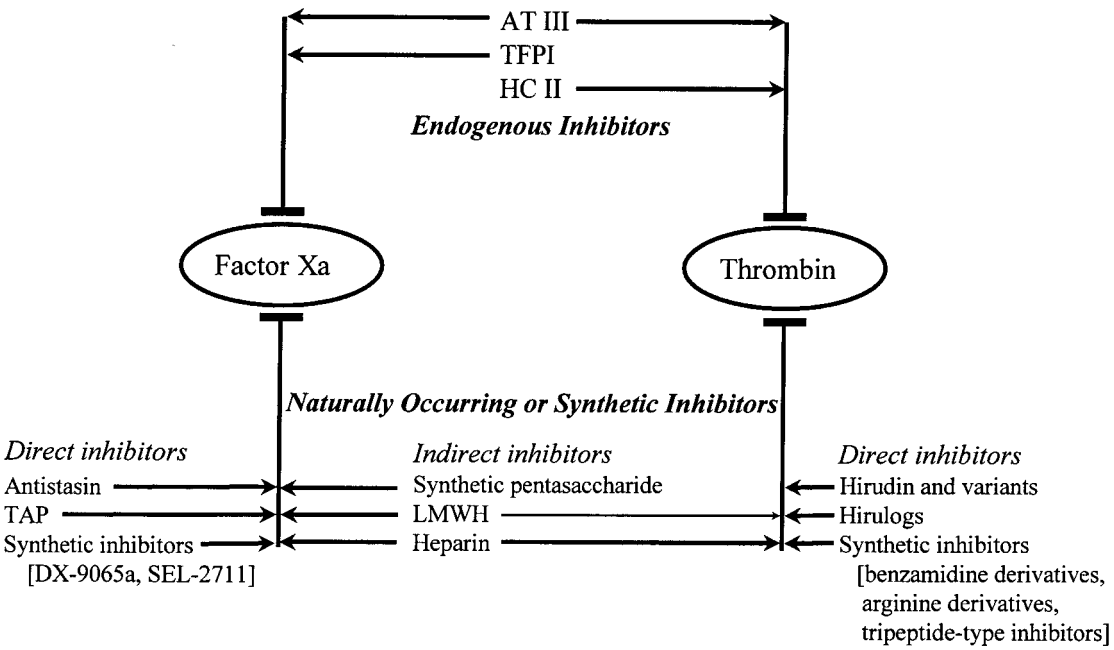

Fig. 2. Various ways of inactivation of thrombin and factor Xa by directly or indirectly acting protease inhibitors.

Table I: Strategies for the inhibition of thrombin.

Potentiation of the endogenous inhibition of thrombin: heparin, LMWH
Direct inactivation of thrombin
Naturally occurring thrombin inhibitors: hirudin (from *Hirudo medicinalis*, recombinant) and triabin (from *Triatoma pallidipennis*, recombinant)
Synthetic active-site inhibitors of thrombin
Irreversible inhibitors: alkylating agents (PPACK (D-Phe-Pro-Arg-CH_2Cl)) and acylating agents (LY-806303)
Reversible covalent inhibitors (transition-state analogs): boronic acid derivatives (DuP-714, SDZ-219349), aldehyde inhibitors (efegatran, CVS-1123) and ketone derivatives (CVS-1347)
Reversible noncovalent inhibitors: arginine derivatives (argatroban) and benzamidine derivatives (NAPAP)
Hirulogs: hirulog-1 (bivalirudin; Hirulog™)

which has been characterized as an anion-binding exosite inhibitor of thrombin (20, 21).

Based on the catalytic mechanism of serine proteinases and the substrate specificity, several types of synthetic thrombin inhibitors have been developed that can be classified according to the kinetics of inhibition and the type of binding or according to the structural origin of the molecule (18, 22-25). Synthetic inhibitors of serine proteinases (Table I) are mainly classified as (i) irreversible alkylating or acylating agents which either alkylate the active-site His^{57} or react with the active-site Ser^{195} to form a stable acyl enzyme intermediate, (ii) reversible covalent inhibitors or transition-state analogs which form inhibitory tetrahedral adducts with the hydroxyl group of the active-site Ser^{195}, such as boronic acids, aldehydes and ketones, and (iii) reversible noncovalent thrombin inhibitors, which are simple substrate analogs such as arginine or benzamidine derivatives (24, 25). Of the many active site-directed compounds developed, the peptide chloromethyl ketone D-Phe-Pro-Arg-CH_2Cl (PPACK) has been found to be a highly potent irreversible inhibitor of thrombin. Among the reversible thrombin inhibitors, especially the arginine derivative argatroban (MD-805), the benzamidine derivative NAPAP, and the tripeptide-type inhibitor efegatran (D-MePhe-Pro-Arg-H) have been shown to be effective and selective inhibitors of thrombin.

In addition, during the last years thrombin inhibitors have been developed that interact with both the active site of the enzyme and the anion-binding exosite. These agents, termed hirulogs, consist of a peptide chain with an anion-binding exosite recognition sequence based on the C-terminus of hirudin, which is linked by glycine residues of variable length to a D-Phe-Pro-Arg-Pro sequence that binds to the active site of thrombin (26-28). Hirulog-1 (bivalirudin, Hirulog™) is a bifunctional 20-amino acid peptide which, via its 12 C-terminal amino acids, interacts with the anion-binding exosite of thrombin, whereas the N-terminal tetrapeptide moiety interacts with the catalytic site of the enzyme (26-28).

Factor Xa inhibitors

There was not a similar rapid development in the field of factor Xa inhibitors as there was for synthetic or naturally occurring thrombin inhibitors. Only a few systematic studies on factor Xa inhibitors comprehensively characterized this class of substances *in vitro* for their inhibitory activity against various coagulation enzymes, as well as *in vivo* for their antithrombotic effectiveness (29). Due to the dramatic increase of the catalytic activity of factor Xa after assembly in the prothrombinase complex an effective factor Xa inhibitor is required to have an extremely high affinity for the enzyme. Synthetic, directly acting factor Xa inhibitors developed and studied in the past had less selectivity over thrombin and, furthermore, the potency of these agents was modest, so that no final conclusions on the anticoagulant/antithrombotic potential of the inhibition of factor Xa could be drawn (30, 31). During the last few years potent factor Xa inhibitors have been isolated from natural sources or were chemically synthesized (Table II) (32). The naturally occurring factor Xa inhibitors are now available as recombinant polypeptides.

Various highly effective and selective polypeptide inhibitors of factor Xa were isolated from blood sucking animals and biochemically and pharmacologically characterized (Table III). Antistasin (ATS), which was isolated from the salivary glands of the Mexican leech *Haementeria officinalis*, is a selective inhibitor of factor Xa which reacts with the active site of the enzyme forming a stable complex (33-36). Tick anticoagulant peptide (TAP) was originally isolated from the tick *Ornithodorus moubata* (37). TAP was shown to be a reversible, slow tight-binding inhibitor of factor Xa. A first low-affinity binding of TAP to a site distinct from the catalytic site is followed by formation of a stable enzyme-inhibitor complex (38, 39). The affinity of TAP for factor Xa assembled in the prothrombinase complex is greater than that for the free enzyme resulting in an appropriately lower inhibition constant (40, 41). Another factor Xa inhibitor, FXaI, was isolated from the saliva of the medicinal leech *Hirudo medicinalis*. A novel recombinant factor Xa inhibitor from *H. medicinalis* is called Yagin (42). Both FXaI and Yagin are slow tight-binding and, except for trypsin, selective factor Xa inhibitors with homologies in their structure and a very similar inhibition pattern. FXaI and Yagin are more effective in inhibiting factor Xa in the prothrombinase complex than the free factor Xa in solution (42). Hematophageous nematodes, such as the hookworm *Ancylostoma caninum*, produce structurally related anticoagulant proteins (nematode anticoagulant proteins, NAP), including effective factor Xa inhibitors (43).

Table II: Strategies for the inhibition of factor Xa.

Potentiation of factor Xa inactivation by endogenous inhibitors: synthetic pentasaccharides
Direct inactivation of factor Xa
Naturally occurring factor Xa inhibitors: antistasin (ATS) (from *Haementeria officinalis*, recombinant), tick anticoagulant peptide (TAP) (from *Ornithodorus moubata*, recombinant) and FXaI/Yagin (from *Hirudo medicinalis*, recombinant)
Synthetic low molecular weight factor Xa inhibitors: DX-9065a and SEL-2711
Inhibition of the activity of the prothrombinase complex: inactive forms of factor Xa

Table III: Characteristics of various inhibitors of factor Xa.

	ATS	TAP	FXaI/Yagin	DX-9065a	SEL-2711
Origin	*Haementeria officinalis* (recombinant)	*Ornithodorus moubata* (recombinant)	*Hirudo medicinalis* (native/recombinant)	Synthetic	Synthetic
Chemical structure	Polypeptide (119 amino acids)	Polypeptide (60 amino acids)	Polypeptide (85 amino acids)	(Amidinoaryl) propanoic acid derivative	Pentapeptide
Molecular weight	15 kDa	6.9 kDa	14.4 kDa	571 Da	760 Da
K_i factor Xa [nmol/l]	0.04-0.06	0.18 (free FXa) 0.006 (FXa complex)	10 (free FXa) 0.12 (FXa complex for FXaI) 0.05 (FXa complex for Yagin)	41	3
Selectivity for factor Xa	High	High	High (except for trypsin)	High	High
Orally active	No	No	No	Yes	Yes
References	33-36	37-41	42	45-48	49, 50

A novel type of synthetic factor Xa inhibitor is DX-9065a. DX-9065a, also called APAP (44), is a non-peptide, low molecular weight compound which has been described as a potent inhibitor of factor Xa with an inhibition constant in the nanomolar range. It is also highly selective for factor Xa and shows oral absorption (45-48). Another synthetic active-site factor Xa inhibitor is SEL-2711 (49, 50). SEL-2711 is a synthetic pentapeptide which is orally available and inhibits factor Xa in a reversible, competitive manner. Some other newly developed synthetic factor Xa inhibitors, such as YM-60828, are in the preclinical phase of development (51-54).

It should be mentioned that, in addition to the direct inhibition of the catalytic activity of the enzyme, factor Xa can also be inactivated by potentiation or acceleration of its inhibition by AT III. Synthetic pentasaccharide, which represents the AT III binding region of heparin, catalyzes the AT III mediated inactivation of factor Xa but not that of thrombin (55, 56).

The inhibition of the activity of the prothrombinase complex by inactive forms of factor Xa (57, 58), which do not have catalytic activity but are still capable of associating into prothrombinase complexes, is at present of more scientific interest. By competitive inhibition of plasma factor Xa for prothrombinase complex assembly by inactive forms, finally thrombin generation is reduced and thrombotic processes can be controlled (58).

Anticoagulant actions

The anticoagulant potency of protease inhibitors both *in vitro* and *in vivo* depends on the mechanism of enzyme inhibition, the affinity to the enzyme, and thus the inhibition constant, as well as on their stability in blood or an interaction with other blood constituents. To measure anticoagulant effects, global clotting assays such as thrombin time (TT), activated partial thromboplastin time (APTT) or prothrombin time (PT) can be used.

Direct thrombin inhibitors are known to exert strong anticoagulant effects *in vitro* as well as *in vivo* due to the inhibition of the catalytic activity of the enzyme (19, 22, 59-62). Blockade of the active site of thrombin prevents not only plasmatic coagulation processes but also thrombin-mediated platelet reactions which may significantly contribute to the antithrombotic effectiveness of these agents. However, platelet activation which is induced by other agonists such as ADP, collagen or arachidonic acid is not inhibited (63). The most sensitive clotting assay for thrombin inhibitors is TT followed by APTT, whereas PT is least sensitive (Fig. 3). The anticoagulant potency of competitive inhibitors correlates to their inhibition constant for thrombin (22).

The inactivation of factor Xa by specific inhibitors does not have any effect on preformed thrombin. However, due to the inhibition of prothrombinase complex assembly, catalytic cleavage of prothrombin and, thus, formation of thrombin is inhibited, as well as the enhancement of thrombin production through autoamplification. Both *in vitro* and *in vivo* naturally occurring or synthetic factor Xa inhibitors did not affect TT, whereas APTT and PT were prolonged in a concentration- or dose-dependent manner (Fig. 3). The factor Xa inhibitors DX-9065a and SEL-2711 demonstrated anticoagulant activities in *ex vivo* blood samples after different routes of

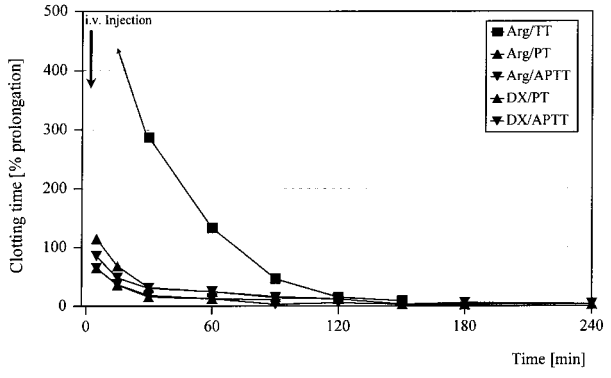

Fig. 3. Anticoagulant action of thrombin (argatroban, Arg, 0.5 mg/kg) and factor Xa (DX-9065a, DX, 1 mg/kg) inhibitors after i.v. bolus injection into rabbits as measured by thrombin time (TT), prothrombin time (PT) and activated partial thromboplastin time (APTT).

administration, including orally, thus showing the oral bioavailability of these compounds (45, 49, 64). The anticoagulant action of some factor Xa inhibitors such as DX-9065a and ATS is species-dependent (34, 65, 66). Furthermore, the anticoagulant potency of the naturally occurring factor Xa inhibitors ATS and TAP did not correlate to their antithrombotic efficacy, *i.e.*, at nearly equal antithrombotic efficiency TAP was less potent than ATS in prolonging APTT which may reflect kinetic differences in the factor Xa inactivation (67). A comparative study on anticoagulant activities of thrombin and factor Xa inhibitors revealed that the PT assay is most sensitive for factor Xa inhibitors (30, 68).

Effect on protease generation

The effect of thrombin and factor Xa inhibitors on the generation of proteases, especially thrombin and factor Xa, is of great interest. Thrombin inhibitors with a high affinity and selectivity for the enzyme are expected to inhibit not only generated thrombin but also thrombin-mediated feedback reactions, such as the activation of cofactors V and VIII, and in this way, to interrupt the amplification mechanisms for thrombin production. On the other hand, the inactivation of factor Xa, which acts at a kinetically important point in the coagulation cascade, by specific inhibitors is assumed to effectively prevent the generation of thrombin. The action of thrombin inhibitors on protease generation was investigated in isolated systems *in vitro* and after administration to humans. At present only few results are available on the influence of factor Xa inhibitors on protease generation.

In biochemically defined systems, thrombin inhibitors such as hirudin or DuP-714 prevented the formation of factor Xa and thrombin after coagulation activation in a concentration-dependent manner (69, 70). Hirudin and hirulog-1 were also shown to inhibit amplification reactions of blood coagulation and, thus, to prevent prothrombin activation (71). In contrast, using platelet-rich plasma, hirudin was less effective in inhibiting thrombin generation and platelet aggregation after intrinsic activation of the coagulation system (72, 73). In whole blood supplemented with hirudin at concentrations that completely prevented clotting of blood a strong increase in levels of the activation markers F1+2 and TAT was found, showing a considerable thrombin generation in the presence of hirudin despite inhibition of clotting (74). The *in vitro* results correlate with *in vivo* findings, where an increased thrombin activity was observed after cessation of therapy with thrombin inhibitors. This rebound phenomenon is thought to be caused by an incomplete and only temporary suppression of thrombin generation by these drugs (75-78). The persistent formation of thrombin may contribute to early rethrombosis and reocclusion after therapy with thrombin inhibitors.

The factor Xa inhibitor DX-9065a was shown to both delay and inhibit thrombin generation in human plasma. Intrinsic thrombin generation was affected to a larger extent than thrombin formation mediated via the extrinsic pathway (64). Recent studies compared the effect of thrombin (hirudin) and factor Xa (DX-9065a) inhibitors on thrombin generation in whole blood after extrinsic or intrinsic activation of the coagulation system (Fig. 4). In the presence of hirudin clotting was prevented, but there was a remarkable generation of thrombin demonstrated by very high F1+2 and TAT levels. The synthetic factor Xa inhibitor was less effective in preventing clotting of blood but was much more active than hirudin in inhibiting thrombin generation. The combination of hirudin with DX-9065a was most effective, *i.e.*, blood clotting was prevented and F1+2 and TAT levels were strongly reduced to normal values. Thus, the combination of a thrombin inhbitor with a factor Xa inhibitor seems to have very favorable effects on the coagulation system, because clotting of blood is prevented by direct inactivation of thrombin and the generation of thrombin is blocked by factor Xa inactivation.

Effect on clot-bound proteases

Intravascular thrombi are known to induce activation of the coagulation system which may play a role, *e.g.*, in rethrombosis and restenosis after coronary thrombolysis. It has been shown that preexisting thrombin which is bound to fibrin in a plasma clot retains its enzymatic activity, and when it is reexposed during fibrinolysis, it is thought to be the primary mediator of thrombus-associated procoagulant activity. Clot-bound thrombin is resistant to inhibition by antithrombin III and the heparin-AT III complex but is susceptible to inactivation by AT III-independent inhibitors such as hirudin or argatroban (79-81). Argatroban as a low molecular weight synthetic thrombin inhibitor was even found to have advantages over hirudin in the inhibitory effect against thrombin bound to a plasma clot when compared to the effect on thrombin in solution. The higher effectiveness of argatroban to inhibit clot-bound thrombin as compared to hirudin is probably based

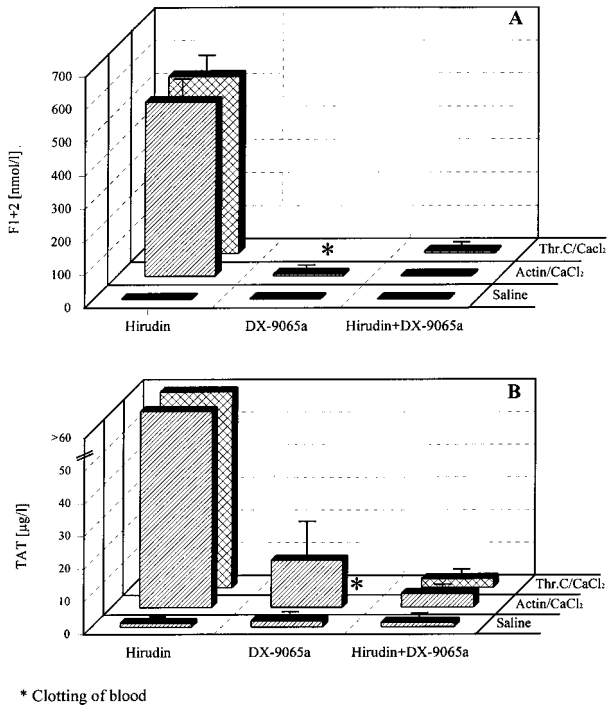

Fig. 4. Comparative effects of thrombin (hirudin, 10 µg/ml final concentration) and factor Xa (DX-9065a, 20 µg/ml final concentration) inhibitors on thrombin generation in whole blood without and after activation of the coagulation system measured by prothrombin fragment F1+2 (A) or thrombin-AT III complexes (B).

on the better accessibility to the clot-fluid interphase as a result of its low molecular weight and on its direct interaction with the active site of thrombin (81).

Newer results indicate that other coagulation factors such as factor Xa are also bound to whole-blood clots. Besides clot-bound thrombin, the procoagulant activity of thrombus-associated factors Xa/Va may be very important for the propagation of intravascular thrombi and for their capability of inducing activation of the coagulation system. In addition to other blood cells, intravascular thrombi contain varying amounts of platelets which may contribute to the procoagulant activity of thrombi in that they provide binding sites for the factor Xa-factor Va complex on their surface, thus promoting the formation of the prothrombinase complex and the resulting activation of prothrombin. Studies on the relative efficacy of inhibitors of thrombin and factor Xa indicate that the thrombus-associated procoagulant activity is mainly dependent on *de novo* activation of prothrombin mediated by factor Xa-factor Va and not primarily on preexisting clot-bound thrombin (82). Clot-associated factor Xa activity was found to be resistant to inhibition by AT III and AT III-dependent inhibitors such as heparin pentasaccharide, but it was inhibited by directly acting factor Xa inhibitors such as TAP and by the endogenous inhibitor tissue factor pathway inhibitor (TFPI), which besides inhibition of the factor VIIa-tissue factor complex, in a first step also binds and inhibits factor Xa (82, 83).

Recent studies, however, have shown that clot-bound factor Xa can be inhibited by both the low molecular weight, synthetic factor Xa inhibitor DX-9065a and the synthetic heparin pentasaccharide SR-90107A/Org-31540 (84). DX-9065a was shown to effectively inhibit prothrombinase activity, whereas hirudin inhibits generated thrombin but is unable to prevent clot-associated prothrombinase activity (85). Because of the presence of clot-associated factor Xa, it can be expected that inhibition of clot-bound thrombin by specific inhibitors is ineffective in preventing continued activation of prothrombin, and that inhibition of factor Xa may attenuate clot-associated procoagulant activity. Thrombin-specific therapy may be effective only as long as it is continued because of the persistence of factor Xa activity (83). This might have important therapeutic implications for the use of inhibitors with a high specificity and selectivity for thrombin. Activation of prothrombin by factor Xa might be responsible, *e.g.*, for the occurrence of recurrent thrombosis after thrombolysis induced by residual thrombi. Thus, it might be concluded that effective inhibition of the procoagulant activity of thrombi requires inactivation of both thrombin and factor Xa (83).

Antithrombotic effects

The formation of thrombi in arterial or venous vessels is a complex process that result from changes in blood flow, in the vessel wall or in blood constituents. Under experimental conditions intravascular thrombosis can be induced by various means such as activation of the coagulation system, endothelial damage of the vessel wall, platelet activation and alterations in blood flow. Thrombin-induced platelet reactions and fibrin formation are essential factors for intravascular thrombus formation. Therefore, the inhibition of thrombin generation and/or the direct inactivation of the enzyme by specific inhibitors are expected to effectively prevent the formation of thrombi and/or their progression.

The antithrombotic effectiveness of thrombin and factor Xa inhibitors was studied in various experimental thrombosis models in which the pathophysiologic mechanisms for the thrombotic process correspond to those which are known to also be relevant in humans. Specific thrombin inhibitors were shown to be potent antithrombotic agents irrespective of the kind of thrombotic challenge, although effective doses and plasma concentrations differed among the various thrombin inhibitors, as well as for a given inhibitor in different thrombosis models (19, 20, 59, 60, 62, 86). Thrombin inhibitors caused dose-dependent antithrombotic actions in both venous and arterial (including coronary) thrombosis, in disseminated intravascular coagulation (DIC) and in arteriovenous shunt thrombosis. They also prevented reocclusion after lysis and increased the efficacy of thrombolytics by shortening the time until lysis. The antithrombotic action was more pronounced in thrombosis models in which the initial generation of thrombin due to activation of the

Table IV: Antithrombotic effectiveness of factor Xa inhibitors in various experimental models of thrombosis (see Refs. 49, 64, 117-119).

Venous thrombosis (rat, rabbit): ATS, TAP, Yagin, DX-9065a, SEL-2711
Arteriovenous shunt thrombosis (rat, baboon): ATS, TAP, Yagin, DX-9065a, SEL-2711
Laser-induced mesenteric microvessel thrombosis (rat): DX-9065a
Acute disseminated intravascular coagulation (mice, rat, rabbit): Yagin, DX-9065a
Coronary artery thrombosis (canine): TAP
Arterial thrombolysis and acute reocclusion after rt-PA (rabbit, canine): ATS, TAP
Restenosis after balloon angioplasty (rabbit): ATS, TAP
Hemodialysis (monkey): DX-9065a

plasmatic coagulation system plays the most important role for the occurrence of vascular thrombi such as in venous thrombosis or DIC. When thrombus formation is primarily initiated by platelet adherence to a damaged area in the vessel wall and, second, by the following activation of plasmatic coagulation, usually higher doses or concentrations of thrombin inhibitors are required for the antithrombotic effect (87-89). Thrombin inhibitors only influence thrombin-mediated platelet reactions and, furthermore, due to the high affinity of thrombin to its receptor on platelets the inhibition of thrombin-induced platelet reactions generally requires higher inhibitor concentrations than the prevention of thrombin-catalyzed fibrinogen conversion (90). For competitive, reversible thrombin inhibitors the antithrombotic potency correlates with their inhibition constants for thrombin.

The inactivation of factor Xa might also be an important antithrombotic principle because of the resulting interference with thrombus formation at an early stage of coagulation activation and because of the persistence of active prothrombinase complexes at the site of vascular injury or inside a thrombus, which may be responsible for the continuation of thrombotic processes during or after treatment with a thrombin inhibitor. Experimental studies carried out with naturally occurring or synthetic factor Xa inhibitors revealed a considerable antithrombotic potential of these compounds in several models of thrombosis, which also included their usefulness for the prevention of arterial thrombosis and as an adjunct to thrombolytic therapy (Table IV). The various factor Xa inhibitors were found to be at least as effective as hirudin or heparin in accelerating reperfusion during thrombolysis and in preventing reocclusion and restenosis. It may be concluded that the inactivation of factor Xa by specific inhibitors and the resulting prevention of thrombin formation may be a promising way to prevent thrombotic processes not only in the venous but also in the arterial system. This class of drugs might be able to prevent mural thrombus formation after arterial injury and the following events that lead to restenosis and reocclusion of the vessel.

Antiproliferative action

Whereas in a normal intact vessel the vascular endothelium provides a nonthrombogenic and nonadhesive surface, after injury and the following changes in endothelial permeabilities, various cellular and molecular processes occur resulting in migration and proliferation of VSMC, formation of a neointima and finally atherosclerotic plaques (91). Platelets, thrombin and other components of the thrombotic process are important factors in neointimal formation (3, 91-93). Thrombin-mediated cellular reactions require the interaction of thrombin with its receptor which is expressed by a variety of cell types including endothelial cells, VSMC, fibroblasts, platelets and macrophages. Thrombin is known to act as a mitogen and/or chemoattractant for these cells (3). Therefore, inactivation of thrombin, interruption of thrombin generation and inhibition of thrombin receptor activation may have potential implications for the inhibition of vascular restenosis. Because the hemostatic and cellular effects of thrombin are of great relevance both for acute vessel closure and late restenosis, thrombin inhibitors have been expected to be suitable agents for the prevention of these processes. The inactivation of thrombin by heparin, which requires AT III for its anticoagulant action, does not effectively inhibit restenosis and reocclusion. This may be based on the inaccessibility of the heparin-AT III complex to its binding site on fibrin-associated thrombin and/or by heparin neutralization by platelet factor 4 released in a platelet-rich thrombus (94). In contrast, direct thrombin inhibitors such as hirudin, hirulog or argatroban are able to penetrate into the thrombus, and, thus, achieve a more potent inhibition of clot-bound thrombin. This may reduce thrombus formation and cellular effects of thrombin at the site of angioplasty.

The antiproliferative action of thrombin inhibitors has been investigated in numerous experimental studies. Direct inactivation of thrombin by specific inhibitors such as hirudin or argatroban, and inhibition of prothrombin activation by factor Xa inhibitors such as TAP, limit neointima formation and prevent or reduce restenosis (94-101). A few clinical studies in patients undergoing coronary angioplasty showed favorable effects of the thrombin inhibitors hirudin, hirulog and argatroban (78, 102-105). However, until now the thrombin inhibitors used in clinical trials were administered only for a relatively short period of time. The outcome after long-term administration of these drugs is still unknown. Furthermore, specific thrombin inhibitors do not effectively block the generation of thrombin (see section on protease generation), so that after cessation of therapy a rebound increase of thrombin activity can occur as observed after withdrawal of argatroban (75), hirudin (76) or hirulog (77, 78).

Studies on mitogenesis using cultured rat VSMC showed that factor Xa is also a potent mitogen which

stimulates DNA synthesis and cell growth (106, 107). Most probably factor Xa exerts its effect indirectly via the PDGF receptor tyrosine kinase pathway. It stimulates VSMC to release preexisting PDGF, which then through the receptor tyrosine kinase pathway leads to the activation of mitogen-activated protein kinases (MAPK) which are well characterized intracellular mediators of cell proliferation (106). This action of factor Xa on VSMC seems to be related to its serine protease activity, since in the presence of specific factor Xa inhibitors such as ATS and TAP the mitogenic effect of factor Xa is blocked (106, 107). Factor Xa-mediated proliferation of VSMC may also be important *in vivo* for the pathogenesis of reocclusion and restenosis after angioplasty. Specific inhibition of factor Xa could limit intimal hyperplasia after damage of the vascular endothelium and, thus, diminish restenosis after balloon angioplasty (108, 109). Experimental studies showed that a 2-hour infusion of ATS significantly reduced restenosis and luminal narrowing by plaque measured 28 days after balloon angioplasty of atherosclerotic femoral arteries in rabbits (108). Administration of TAP for 60 hours resulted in a long-term decrease in neointimal thickness of porcine coronary arteries 28 days after severe injury (109). The results indicate that specific factor Xa inhibitors might be effective substances to reduce neointimal hyperplasia either by preventing the mitogenic effects of factor Xa and/or by inhibiting the generation of thrombin as a potent mitogen.

Clinical implications

The principal criteria for the clinical use of thrombin and factor Xa inhibitors are their effectiveness and safety. Preclinical studies with both classes of serine proteinase inhibitors showed that the direct inactivation of coagulation enzymes by specific inhibitors is an effective approach for anticoagulant/antithrombotic therapy.

Recent clinical trials suggest that active site-directed thrombin inhibitors might be useful for prophylaxis and/or therapy of various thromboembolic disorders. Among the numerous thrombin inhibitors known, particularly hirudin, PEG-hirudin, hirulog and argatroban have been studied for various clinical indications. The clinical development of these agents has been reviewed recently (110-115). Thrombin inhibitors are the most promising class of drugs for the initial treatment of patients with heparin-induced thrombocytopenia (HIT). Hirudin (Refludan®, Revasc®) and argatroban (Novastan®) have been investigated or approved for their use as alternate anticoagulants in HIT patients (116). Completed or ongoing trials with thrombin inhibitors were reported in cardiovascular indications such as unstable angina, percutaneous coronary interventions and acute myocardial infarction (110, 114, 115). The clinical trials have shown a decreased risk of acute ischemic complications following PTCA, but only a modest benefit over heparin in acute coronary syndromes or when used as adjunct drugs to thrombolytic agents. Thrombin inhibitors such as hirudin or hirulog have also been found to be safe and efficacious in the management of postoperative venous thrombosis after knee or hip surgery (27, 111). Possible indications for the clinical use of thrombin inhibitors are given in Table V.

Table V: Possible clinical indications for thrombin inhibitors.

Replacement of heparin in patients with HIT or AT III deficiency
Prevention of restenosis following angioplasty or thrombolysis
Adjunctive administration in thrombolysis
Postoperative venous thrombosis prophylaxis
Anticoagulation in hemodialysis and extracorporeal circulation
Disseminated intravascular coagulation

Most of the specific factor Xa inhibitors known at the present time are still in the phase of preclinical development or are being investigated in first clinical studies. Based on experimental findings, factor Xa inhibitors are expected to be suitable agents for the prophylaxis and/or treatment of various thrombotic disorders. Due to the inhibition of the generation of thrombin, factor Xa inhibitors could be very useful agents for the prophylaxis of less severe thrombotic processes such as venous thromboembolism. The inactivation of clot-associated prothrombinase activity by low molecular, specific factor Xa inhibitors which are able to penetrate into the clot may be a valuable tool to prevent the perpetuation of thrombotic processes after angioplasty or thrombolysis. Thus, these agents might have important therapeutic implications in coronary and cerebrovascular ischemic syndromes such as coronary thrombosis, unstable angina and stroke. They may be useful in preventing reocclusion and restenosis after PTCA, as adjuncts to thrombolytic therapy or in combination with new antiplatelet agents such as clopidogrel or GPIIb/IIIa inhibitors. However, the real potential of factor Xa inhibitors has still to be validated in comprehensive clinical trials.

An important point is that factor Xa inhibitors cannot interrupt thrombotic processes which are caused by generated thrombin. Therefore, to effectively suppress the procoagulant activity of intravascular thrombi and the progression of thrombosis, it may be a promising approach to inhibit both thrombin and factor Xa by respective inhibitors. This may provide therapeutic benefit in a range of clinical indications.

A major side effect of anticoagulant/antithrombotic therapy are bleeding complications which often limit the therapeutic value of coagulation enzyme inhibitors. In animal experiments thrombin inhibitors were found to prolong the bleeding time in a dose-dependent manner. However, under experimental conditions primary hemostasis was markedly influenced only at dosages which were much higher than those required for antithrombotic effects (88, 89). In healthy volunteers, thrombin inhibitors showed no evidence of bleeding complications and did not prolong the bleeding time (110). In contrast, bleeding complications occurred in clinical trials with hirudin for the treatment of myocardial infarction (110, 111, 113). This

Table VI: Characteristics of direct thrombin inhibitors vs. *direct factor Xa inhibitors.*

	Thrombin Inhibitors	Factor Xa Inhibitors
Target enzyme	Specific and potent inhibition of thrombin	Specific and potent inhibition of factor Xa
Anticoagulant action	TT assay is most sensitive	PT and APTT assays are most sensitive
Effect on proteases and their generation	Inhibition of generated thrombin, no inhibition of protease generation	Inhibition of protease generation, no inhibition of preexisting thrombin
Effect on clot-associated coagulation enzymes	Inhibition of clot-bound thrombin	Inhibition of clot-bound factor Xa
Antihrombotic action in various experimental models	Yes	Yes
Hemorrhagic effects	Bleeding complications	High benefit/risk ratio, no bleeding side effects
Induction of thrombocytopenia	No	No
Interaction with other factors	Numerous interactions with other structures (due to multiple functions of thrombin)	Fewer interactions with other plasmatic and cellular components

might be due to an inadequate dose regimen or adjustment or due to interactions with other drugs such as aspirin. Other trials revealed no differences in major or minor bleedings among groups receiving heparin or hirudin, and furthermore, in prevention of postoperative venous thromboembolism after orthopedic surgery, hirudin was more effective than heparin without increase in bleeding risk (113). However, the results from clinical trials suggest that there is a relatively narrow therapeutic window for hirudin. Until now, hirulog showed a high effectiveness in patients with unstable angina and after PTCA without any increased risk of bleeding complications (27, 111, 113).

Factor Xa inhibitors such as DX-9065a were antithrombotically effective at doses that did not affect bleeding time or blood loss (29, 45, 117). For some reasons, it can be expected that factor Xa inhibitors express a better safety/efficacy profile than heparin or direct thrombin inhibitors. Factor Xa inhibitors do not impair primary hemostasis because they do not affect platelet aggregation. As competitive inhibitors they do not completely suppress thrombin formation. Small amounts of thrombin generated at a site of vascular injury are able to initiate the formation of a hemostatic plug but cannot catalyze the conversion of fibrinogen into fibrin in amounts which are sufficient to form an intravascular thrombus.

Conclusions

The development of direct inhibitors of serine proteinases with high specificity and selectivity provides new means for effectively controlling blood coagulation processes and intravascular thrombus formation. Direct thrombin and direct factor Xa inhibitors both have potential advantages over heparin and heparin-related drugs in that they are able to penetrate into a thrombus to inactivate clot-associated thrombin as well as factor Xa, and furthermore, their use is not limited by the occurrence of an antibody-based thrombocytopenia syndrome. Despite differences in the site and mechanism of action and the resulting effects on the coagulation system (Table VI), these agents reflect newer therapeutic strategies and can provide therapeutic benefit in various clinical indications. Currently available thrombin and factor Xa inhibitors represent different classes of drugs with great chemical and functional heterogeneity. Therefore, each of these compounds should be considered and characterized as a distinct drug.

Despite major progress in the development of antithrombin and antifactor Xa agents, there are still some unresolved issues. The principal limitations of thrombin inhibitors result from their potential to cause bleeding complications and the lack of an available antidote at the present time, as well as from their ineffectiveness in inhibiting protease generation which may be responsible for the rebound phenomenon after cessation of antithrombotic therapy. Furthermore, pharmacokinetic characteristics such as a relatively short half-life (except for PEG-hirudin) or the poor oral bioavailability must be taken into consideration. On the other hand, the short duration of the anticoagulant effect might be advantageous in case of an overdose when no antagonist is required for neutralization.

With regard to factor Xa inhibitors, these agents strongly inhibit the formation of thrombin and thus can prevent or even interrupt the thrombotic process, although they are expected to be much less antithrombotically effective when sufficient amounts of thrombin have already been generated. Due to their mechanism of action, competitive factor Xa inhibitors are expected not to cause hemorrhagic side effects such as those seen with thrombin inhibitors.

In addition to the anticoagulant/antithrombotic effectiveness, a general assessment of the therapeutic potential of a given drug should include other important aspects such as metabolic transformations, excretory routes, interactions with other drugs or endogenous factors, as

well as additional mechanisms of actions. A particular thrombin or factor Xa inhibitor might be useful for only a specific clinical indication, and it is likely that one drug might not be the optimum treatment for all thrombotic situations. Furthermore, since only the inhibition of thrombin may not always be sufficient to effectively interrupt thrombotic mechanisms which are related to restenosis and reocclusion processes, it is necessary to analyze whether the combination of thrombin with factor Xa inhibition would be a useful strategy to suppress procoagulant activity *in vivo*. Additional investigations and comprehensive clinical trials are needed to demonstrate the inhibitory profile, the effectiveness or the superiority of thrombin over factor Xa inhibitors and *vice versa* in thrombotic and cardiovascular indications.

Acknowledgements

The author gratefully acknowledges the generous gift of DX-9065a from Dr. S. Kunitada of Daiichi (Tokyo, Japan).

References

1. Fenton, J.W.II. *Thrombin functions and antithrombotic intervention.* Thromb Haemost 1995, 74: 493-8.

2. Stubbs, M.T., Bode, W. *A player of many parts: The spotlight falls on thrombin's structure.* Thromb Res 1993, 69: 1-58.

3. Kanthou, C., Benzakour, O. *Cellular effects of thrombin and their signalling pathways.* Cell Pharmacol 1995, 2: 293-302.

4. Esmon, C.T. *Cell mediated events that control blood coagulation and vascular injury*. Annu Rev Cell Biol 1993, 9: 1-26.

5. Vu, T.K.H., Hung, D.T., Wheaton, V.I., Coughlin, S.R. *Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation.* Cell 1991, 64: 1057-68.

6. Brinkman, H.J.M., Mertens, K., Holthuis, J., Zwarthuinink, L.A., Grijm, K., Vanmourik, J.A. *The activation of human blood coagulation factor X on the surface of endothelial cells: A comparison with various vascular cells, platelets and monocytes.* Br J Haematol 1994, 87: 332-42.

7. Mann, K.G. *The assembly of blood clotting complexes on membranes.* Trends Biochem Sci 1987, 12: 229-33.

8. Mann, K.G., Kalafatis, M. *The coagulation explosion.* Cerebrovasc Dis 1995, 5: 93-7.

9. Nicholson, A.C., Nachman, R.L., Altieri, D.C. et al. *Effector cell protease receptor-1 is a vascular receptor for coagulation factor Xa.* J Biol Chem 1996, 271: 28407-13.

10. Bono, F., Herault, J.P., Avril, C., Schaeffer, P., Lormeau, J.C., Herbert, J.M. *Human umbilical vein endothelial cells express high affinity receptors for factor Xa.* J Cell Physiol 1997, 172: 36-43.

11. Bouchard, B.A., Catcher, C.S., Thrash, B.R., Adida, C., Tracy, P.B. *Effector cell protease receptor-1, a platelet activation-dependent membrane protein, regulates prothrombinase-catalyzed thrombin generation*. J Biol Chem 1997, 272: 9244-51.

12. Rosenberg, R.D., Rosenberg, J.S. *Natural anticoagulant mechanisms*. J Clin Invest 1984, 74: 1-6.

13. Mann, K.G., Nesheim, M.E., Church, W.R. Haley, P., Krishnaswamy, S. *Surface-dependent reactions of the vitamin K-dependent enzyme complexes.* Blood 1990, 76: 1-16.

14. Tollefsen, D.M., Majerus, D.W., Blank, M.K. *Heparin cofactor II. Purification and properties of a heparin-dependent inhibitor of thrombin in human plasma.* J Biol Chem 1982, 257: 2162-9.

15. Dahlbäck, B. *The protein C anticoagulant system: Inherited defects as basis for venous thrombosis.* Thromb Res 1995, 77: 1-43.

16. Broze, G.J. Jr. *Tissue factor pathway inhibitor and the current concept of blood coagulation.* Blood Coag Fibrinol 1995, 6: S7-13.

17. Fenton, J.W. II. *Thrombin bioregulatory functions.* Adv Clin Enzymol 1988, 6: 186-93.

18. Talbot, M.D., Butler, K.D. *Potential clinical uses of thrombin inhibitors.* Drug News Perspect 1990, 3: 357-63.

19. Callas, D., Fareed J. *Comparative pharmacology of site directed antithrombin agents. Implication in drug development.* Thromb Haemost 1995, 74: 473-81.

20. Noeske-Jungblut, C., Haendler, B., Donner, P., Alagon, A., Possani, L., Schleuning, W.D. *Triabin: A highly potent exosite inhibitor of thrombin*. J Biol Chem 1995, 270: 28629-34.

21. Glusa, E., Bretschneider, E., Daum, J., Noeske-Jungblut, C. *Inhibition of thrombin-mediated cellular effects by triabin, a highly potent anion-binding exosite thrombin inhibitor.* Thromb Haemost 1997, 77: 1196-200.

22. Hauptmann, J., Markwardt, F. *Pharmacological aspects of the development of selective synthetic thrombin inhibitors as anticoagulants*. Semin Thromb Hemost 1992, 18: 200-17.

23. Markwardt, F., Stürzebecher, J. *Inhibitors of trypsin and trypsin-like enzymes with a physiological role.* In: Design of Enzyme Inhibitors as Drugs. Sandler, M., Smith, H.J. (Eds.). Oxford University Press: Oxford 1989, 619-49.

24. Shafer, J.A. *Direct thrombin inhibitors: Active-site inhibitors of thrombin.* In: New Therapeutic Agents in Thrombosis and Thrombolysis. Sasahara, A.A., Loscalzo, J. (Eds.). Marcel Dekker, Inc.: New York 1997, 143-57.

25. Powers, J.C., Kam, C.M. *Synthetic substrates and inhibitors of thrombin.* In: Thrombin: Structure and Function. Berliner, L.J. (Ed.). Plenum Press: New York 1992, 117-58.

26. Maraganore, J.M., Bourdon, P., Jablonski, J., Ramachandran, K.L., Fenton, J.W. II. *Design and characterization of hirulogs: A novel class of bivalent peptide inhibitors of thrombin*. Biochemistry 1990, 29: 7095-101.

27. Maraganore, J.M. *Direct thrombin inhibitors: Hirulog.* In: New Therapeutic Agents in Thrombosis and Thrombolysis. Sasahara, A.A., Loscalzo, J. (Eds.). Marcel Dekker, Inc.: New York 1997, 191-208.

28. Fenton, J.W. II, Ni, F., Witting, J.I., Brezniak, D.V., Andersen, T.T., Malik, A.B. *The rational design of thrombin-directed antithrombotics*. In: The Design of Synthetic Inhibitors of

Thrombin. Claeson, G. (Ed.). Plenum Press: New York 1993, 1-13.

29. Kaiser, B., Hauptmann, J. *Factor Xa inhibitors as novel antithrombotic agents: Facts and perspectives.* Cardiovasc Drug Rev 1994, 12: 225-36.

30. Hauptmann, J., Kaiser, B., Nowak, G., Stürzebecher, J., Markwardt, F. *Comparison of the anticoagulant and antithrombotic effects of synthetic thrombin and factor Xa inhibitors.* Thromb Haemost 1990, 63: 220-3.

31. Stürzebecher, J., Stürzebecher, U., Vieweg, H., Wagner, G., Hauptmann, J., Markwardt, F. *Synthetic inhibitors of bovine factor Xa and thrombin. Comparison of their anticoagulant efficiency.* Thromb Res 1989, 54: 245-52.

32. Ripka, W., Brunck, T., Stanssens, P. et al. *Strategies in the design of inhibitors of serine proteases of the coagulation cascade – Factor Xa.* Eur J Med Chem 1995, 30 (Suppl.): 88-100s.

33. Nutt, E., Gasic, T., Rodkey, J. et al. *The amino acid sequence of antistasin.* J Biol Chem 1988, 263: 10162-7.

34. Nutt, E.M., Jain, D., Lenny, A.B., Schaffer, L., Siegl, P.K., Dunwiddie, C.T. *Purification and characterization of recombinant antistasin: A leech-derived inhibitor of coagulation factor Xa.* Arch Biochem Biophys 1991, 285: 37-44.

35. Tuszynski, G.P., Gasic, T.B., Gasic, G.J. *Isolation and characterization of antistasin. An inhibitor of metastasis and coagulation.* J Biol Chem 1987, 262: 9718-23.

36. Dunwiddie, C.T., Thornberry, N.A., Bull, H.G. et al. *Antistasin, a leech-derived inhibitor of factor Xa. Kinetic analysis of enzyme inhibition and identification of the reactive site.* J Biol Chem 1989, 264: 16694-9.

37. Waxman, L., Smith, D.E., Arcuri, K.E., Vlasuk, G.P. *Tick anticoagulant peptide (TAP) is a novel inhibitor of blood coagulation factor Xa.* Science 1990, 248: 593-6.

38. Jordan, S.P., Mao, S.S., Lewis, S.D., Shafer, J.A. *Reaction pathway for inhibition of blood coagulation factor Xa by tick anticoagulant peptide.* Biochemistry 1992, 31: 5374-80.

39. Jordan, S.P., Waxman, L., Smith, D.E., Vlasuk, G.P. *Tick anticoagulant peptide: Kinetic analysis of the recombinant inhibitor with blood coagulation factor Xa.* Biochemistry 1990, 29: 11095-100.

40. Krishnaswamy, S., Vlasuk, G.P., Bergum, P.W. *Assembly of the prothrombinase complex enhances the inhibition of bovine factor Xa by tick anticoagulant peptide.* Biochemistry 1994, 33: 7897-907.

41. Vlasuk, G.P. *Structural and functional characterization of tick anticoagulant peptide (TAP): A potent and selective inhibitor of blood coagulation factor Xa.* Thromb Haemost 1993, 70: 212-6.

42. Rigbi, M., Jackson, C.M., Atamna, H. et al. *FXa inhibitor from the saliva of the leech Hirudo medicinalis.* Thromb Haemost 1995, 73: 1306.

43. Vlasuk, G.P., Bergum, P.W., Brunck, T.K. et al. *Anticoagulant repertoire of hematophagous nematodes.* Thromb Haemost 1995, 73: 1305.

44. Yokoyama, T., Kelly, A.B., Marzec, U.M., Hanson, S.R., Kunitada, S., Harker, L.A. *Antithrombotic effects of orally active synthetic antagonists of activated factor X in nonhuman primates.* Circulation 1995, 92: 485-91.

45. Hara, T., Yokoyama, A., Ishihara, H., Yokoyama, Y., Nagakara, T., Iwamoto, M. *DX-9065a, a new synthetic, potent anticoagulant and selective inhibitor for factor Xa.* Thromb Haemost 1994, 71: 314-9.

46. Katakura, S., Nagakara, T., Hara, T., Iwamoto, M. *A novel factor Xa inhibitor: Structure-activity relationships and selectivity between factor Xa and thrombin.* Biochem Biophys Res Commun 1993, 197: 965-72.

47. Katakura, S., Nagakara, T., Hara, T., Kunitada, S., Iwamoto, M. *Molecular model of an interaction between factor Xa and DX-9065a, a novel factor Xa inhibitor: Contribution of the acetimidoylpyrrolidine moiety of the inhibitor to potency and selectivity for serine proteases.* Eur J Med Chem 1995, 30: 387-94.

48. Nagahara, T., Katakura, S., Yokoyama, Y. et al. *Design, synthesis and biological activities of orally active coagulation factor Xa inhibitors.* Eur J Med Chem 1995, 30 (Suppl.): 140-3s.

49. Seligman, B., Stringer, S.K., Ostrem, J.A. et al. *SEL 2711: A specific, orally available, active-site inhibitor of factor Xa discovered using synthetic combinatorial chemistry.* Presented at the 6th IBC International Symposium: Advances in Anticoagulants & Antithrombotics, Washington, DC 1995.

50. Ostrem, J.A., Stringer, S., Al-Obedi, F. et al. *Characterization of an orally available and highly specific synthetic factor Xa inhibitor.* Thromb Haemost 1995, 73: 1306.

51. Taniuchi, Y., Sakai, Y., Kawasaki, T. et al. *Anticoagulant and pharmacokinetic profiles of YM-60828, an orally active and potent synthetic inhibitor of factor Xa.* Thromb Haemost 1997; Suppl.: 294.

52. Sato, K., Kawasaki, T., Hisamichi, N. et al. *A selective and potent factor Xa inhibitor, YM-60828, exerts its antithrombotic effect with little prolongation of bleeding time.* Thromb Haemost 1997, Suppl.: 292.

53. Gunn, A.C., Wong, P.W., Marlowe, C.K., Zhu, B.Y., Scarborough, R.M., Sinha, U. *Design, synthesis and characterization of specific inhibitors of coagulation factor Xa.* Thromb Haemost 1997, Suppl.: 294.

54. Wong, A.G., Ku, P., Lin, P.H. et al. *Inhibition of rabbit deep vein thrombosis by specific inhibitors of coagulation factor Xa.* Thromb Haemost 1997, Suppl.: 293.

55. Lormeau, J.C., Herault, J.P. *The effect of the synthetic pentasaccharide SR 90107/Org 31540 on thrombin generation ex vivo is uniquely due to ATIII-mediated neutralization of factor Xa.* Thromb Haemost 1995, 74: 1474-7.

56. Olson, S.T., Björk, I., Sheffer, R., Craig, P.A., Shore, J.D., Choay, J. *Role of antithrombin-binding pentasaccharide in heparin acceleration of antithrombin-proteinase reactions.* J Biol Chem 1992, 267: 12528-38.

57. Benedict, C.R., Ryan, J., Todd, J. et al. *Active site-blocked factor Xa prevents thrombus formation in the coronary vasculature in parallel with inhibition of extravascular coagulation in a canine thrombosis model.* Blood 1993, 81: 2059-66.

58. Sinha, U., Hollenbach, S., Wolf, D.L. *Macromolecu-lar complex assembly of prothrombinase is a central process of thrombosis.* In: Ann NY Acad Sci, Vol. 714: Platelet-Dependent Vascular Occlusion. G.A. FitzGerald, L.K. Jennings, C. Patrono (Eds.). New York 1994, 32-40.

59. Bush, L.R. *Argatroban, a selective, potent thrombin inhibitor.* Cardiovasc Drug Rev 1991, 9: 247-63.

60. Kaiser, B. *Anticoagulant and antithrombotic actions of recombinant hirudin.* Semin Thromb Hemost 1991, 2: 130-6.

61. Walsmann, P., Kaiser, B. *Biochemical and pharmacological properties of recombinant hirudin.* Drugs Today 1989, 25: 473-85.

62. Kaiser, B., Hauptmann, J. *Pharmacology of synthetic thrombin inhibitors of the tripeptide type.* Cardiovasc Drug Rev 1992, 10: 71-87.

63. Kaiser, B., Koza, M., Walenga, J.M., Fareed, J. *Flow cytometric evaluation of the effect of various thrombin inhibitors on platelet activation in whole blood.* Thromb Res 1996, 82; 257-63.

64. Herbert, J.M., Bernat, A., Dol, F., Hérault, J.P., Crépon, B., Lormeau, J.C. *DX 9065A, a novel, synthetic, selective, and orally active inhibitor of factor Xa: In vitro and in vivo studies*. J Pharmacol Exp Ther 1996, 276: 1030-8.

65. Hara, T., Yokoyama, A., Morishima, Y., Kunitada, S. *Species differences in anticoagulant and anti-Xa activity of DX-9065a, a highly selective factor Xa inhibitor.* Thromb Res 1995, 80: 99-104.

66. Hauptmann, J., Kaiser, B. *Anticoagulant and antithrombotic action of the factor Xa inhibitor antistasin (ATS).* Thromb Res 1993, 71: 169-74.

67. Vlasuk, G.P., Ramjit, D., Fujita, T. et al. *Comparison of the in vivo anticoagulant properties of standard heparin and the highly selective factor Xa inhibitors antistasin and tick anticoagulant peptide (TAP) in a rabbit model of venous thrombosis.* Thromb Haemost 1991, 65: 257-62.

68. Hauptmann, J., Kaiser, B. *Anticoagulant potential of synthetic and recombinant inhibitors of factor Xa and thrombin in vitro.* Blood Coag Fibrinol 1993, 4: 577-82.

69. Kaiser, B., Fareed, J., Hoppensteadt, D., Birdsong, B., Walenga, J.M., Markwardt, F. *Influence of recombinant hirudin and unfractionated heparin on thrombin and factor Xa generation in extrinsic and intrinsic activated systems.* Thromb Res 1992, 65: 157-64.

70. Kaiser, B., Callas, D., Hoppensteadt, D., Mallinows-ka, K., Fareed, J. *Comparative studies on the inhibitory spectrum of recombinant hirudin, DuP 714 and heparin on thrombin and factor Xa generation in biochemically defined systems.* Thromb Res 1994, 73: 327-35.

71. Ofosu, F.A., Fenton, J.W. II, Maraganore, J. et al. *Inhibition of the amplification reactions of blood coagulation by site-specific inhibitors of α-thrombin*. Biochem J 1992, 283: 893-7.

72. Gallistl, S., Muntean, W. *Thrombin-hirudin complex formation, thrombin-antithrombin III complex formation, and thrombin generation after intrinsic activation of plasma.* Thromb Haemost 1994, 72: 387-92.

73. Gallistl, S., Muntean, W., Leis, H.J. *Effects of heparin and hirudin on thrombin generation and platelet aggregation after intrinsic activation of platelet rich plasma.* Thromb Haemost 1995, 74: 1163-8.

74. Kaiser, B., Fareed, J., Walenga, J.M., Hoppensteadt, D., Markwardt, F. *In vitro studies on thrombin generation in citrated, r-hirudinized and heparinized whole blood.* Thromb Res 1991, 64: 589-96.

75. Gold, H.K., Torres, F.W., Garabedian, H.D. et al. *Evidence for a rebound coagulation phenomenon after cessation of a 4-hour infusion of a specific thrombin inhibitor in patients with unstable angina pectoris.* J Am Coll Cardiol 1993, 21: 1039-47.

76. Zoldhelyi, P., Bichler, J., Owen, W.G. et al. *Persistent thrombin generation in humans during specific thrombin inhibition with hirudin.* Circulation 1994, 90: 2671-8.

77. Ginsberg, J.S., Nurmohamed, M.T., Gent, M. et al. *Effects on thrombin generation of single injections of Hirulog™ in patients with calf vein thrombosis.* Thromb Haemost 1994, 72: 523-5.

78. Bittl, J.A., Strony, J., Brinker, J.A. et al. *Treatment with bivalirudin (Hirulog) as compared with heparin during coronary angioplasty for unstable or postinfarction angina.* New Engl J Med 1995, 333: 764-9.

79. Mirshahi, M., Soria, J., Soria, C. et al. *Evaluation of the inhibition by heparin and hirudin of coagulation activation during r-t-PA-induced thrombolysis.* Blood 1989, 74: 1025-30.

80. Weitz, J.I., Hudoba,, M., Massel, D., Maraganore, J., Hirsh, J. *Clot-bound thrombin is protected from inhibition by heparin-antithrombin III but is susceptible to inactivation by antithrombin III-independent inhibitors*. J Clin Invest 1990, 86: 385-91.

81. Berry, C.N., Girardot, C., Lecoffre, C., Lunven, C. *Effects of the synthetic thrombin inhibitor argatroban on fibrin- or clot-incorporated thrombin: Comparison with heparin and recombinant hirudin.* Thromb Haemost 1994, 72: 381-6.

82. Prager, N.A., Abendschein, D.R., McKenzie, C.R., Eisenberg, P.R. *Role of thrombin compared with factor Xa in the procoagulant activity of whole blood clots.* Circulation 1995, 92: 962-7.

83. Eisenberg, P.R., Siegel, J.E., Abendschein, D.R., Miletich, J.P. *Importance of factor Xa in determining the procoagulant activity of whole-blood clots.* J Clin Invest 1993, 91: 1877-83.

84. Hérault, J.P., Pflieger, A.M., Bernat, A., Lormeau, J.C., Herbert, J.M. *Comparative effects of two factor Xa inhibitors: The synthetic pentasaccharide SR 90107 A/Org 31540 and DX 90675 on free and clot-bound prothrombinase.* Thromb Haemost 1997, Suppl.: 293.

85. Meddahi, S., Bara, L., Fessi, H., Samama, M.M. *Evaluation of clot-associated procoagulant activities in the presence of DX-9065a, rhirudin, enoxaparin and heparin: By a chromogenic technique, F1+2 and TAT measurements.* Thromb Haemost 1997, Suppl.: 294.

86. Berry, C.N., Girard, D., Girardot, C., Lochot, S., Lunven, C., Visconte, C. *Antithrombotic activity of argatroban in experimental thrombosis in the rabbit.* Semin Thromb Hemost 1996, 22: 233-41.

87. Berry, C.N., Girard, D., Lochot, S., Lecoffre, C. *Antithrombotic actions of argatroban in rat models of venous, 'mixed' and arterial thrombosis, and its effect on the tail transection bleeding time.* Br J Pharmacol 1994, 113: 1209-14.

88. Markwardt, F., Kaiser, B., Nowak, G. *Studies on antithrombotic effects of recombinant hirudin.* Thromb Res 1989, 54: 377-88.

89. Kaiser, B., Markwardt, F. *Experimental studies on the antithrombotic action of a highly effective, synthetic thrombin inhibitor*. Thromb Haemost 1986, 55: 194-6.

90. Hoffmann, A., Markwardt, F. *Inhibition of the thrombin-platelet reaction by hirudin.* Haemostasis 1984, 14: 164-9.

91. Benzakour, O., Kanthou, C. *Cellular and molecular events in atherogenesis: basis for pharmacological and gene therapy approaches to restenosis.* Cell Pharmacol 1996, 3: 7-22.

92. Schwartz, R.S., Holmes, D.R. Jr., Topol, E.J. *The restenosis paradigm revisited: an alternative proposal for cellular mechanisms.* J Am Coll Cardiol 1992; 20: 1284-1293.

93. McNamara, C.A., Sarembock, I.J., Bachhuber, B.G. et al. *Thrombin and vascular smooth muscle cell proliferation: Implications for atherosclerosis and restenosis.* Semin Thromb Hemost 1996, 22: 139-44.

94. Ali, M.N., Villarreal-Levy, G., Schafer, A.I. *The role of thrombin and thrombin inhibitors in coronary angioplasty*. Chest 1995, 108: 1409-19.

95. Sarembock, I.J., Gertz, S.D., Gimple, L.W., Owen, R.M., Powers, E.R., Roberts, W.C. *Effectiveness of recombinant desulphatohirudin in reducing restenosis after balloon angioplasty of atherosclerotic femoral arteries in rabbits.* Circulation 1991, 84: 232-43.

96. Heras, M., Chesebro, J.H., Webster, M.W.I. et al. *Hirudin, heparin, and placebo during deep arterial injury in the pig: the in vivo role of thrombin in platelet-mediated thrombosis.* Circulation 1990, 82: 1476-84.

97. Kaiser, B., Simon, A., Markwardt, F. *Antithrombotic effects of recombinant hirudin in experimental angioplasty and intravascular thrombolysis*. Thromb Haemost 1990, 63: 44-7.

98. Imanishi, T., Arita, M., Hamada, M., Tomobuchi, Y., Hano, T., Nishio, I. *Effects of locally administration of argatroban using a hydrogel-coated balloon catheter on intimal thickening induced by balloon injury.* Jpn Circ J 1997, 61: 256-62.

99. Abendschein, D.R., Recchia, D., Meng, Y.Y., Oltrona, L., Wickline, S.A., Eisenberg, P.R. *Inhibition of thrombin attenuates stenosis after arterial injury in minipigs.* J Am Coll Cardiol 1996, 28: 1849-55.

100. Unterberg, C., Sandrock, D., Nebendahl, K., Buchwald, A.B. *Reduced acute thrombus formation results in decreased neointimal proliferation after coronary angioplasty.* J Am Coll Cardiol 1995, 26: 1747-54.

101. Barry, W.L., Wiegman, P.J., Gimple, L.W. et al. *A new single-injury model of balloon angioplasty in cholesterol-fed rabbits: Beneficial effect of hirudin and comparison with double-injury model.* Lab Invest 1997, 77: 109-16.

102. Van de Boos, A.A., Deckers, J.W., Heyndrickx, G.R. et al. *Safety and efficacy of recombinant hirudin (CGP 39393) versus heparin in patients with stable angina undergoing coronary angioplasty.* Circulation 1993, 88: 2058-66.

103. Topol, E.J., Bonan, R., Jewitt, D. et al. *Use of a direct antithrombin, hirulog, in place of heparin during coronary angioplasty*. Circulation 1993, 87: 1622-9.

104. Serryus, P.W., Herrman, J.P.R., Simon, R. et al. *A comparison of hirudin with heparin in the prevention of restenosis after coronary angioplasty.* New Engl J Med 1995, 333: 757-63.

105. Herrman, J.P.R., Suryapranata, H., den Heijer, P., Kutryk, M.J.B., Gabriël, L., Serryus, P.W. *Argatroban during percutaneous transluminal angioplasty: Results of a dose verification study.* J Thromb Thrombolysis 1996, 3: 367-75.

106. Ko, F.N., Yang, Y.C., Huang, S-.C., Ou, J.T. *Coagulation factor Xa stimulates platelet-derived growth factor release and mitogenesis in cultured vascular smooth muscle cells of rat.* J Clin Invest 1996, 98: 1493-501.

107. Gasic, G.P., Arenas, C.P., Gasic, T.B., Gasic, G.J. *Coagulation factors X, Xa, and protein S as potent mitogens of cultured aortic smooth muscle cells.* Proc Natl Acad Sci USA 1992, 89: 2317-20.

108. Ragosta, M., Gimple, L.W., Gertz, D. et al. *Specific factor Xa inhibition reduces restenosis after balloon angioplasty of atherosclerotic femoral arteries in rabbits.* Circulation 1994, 89: 1262-71.

109. Schwartz, R.S., Holder, D.J., Holmes, D.R. Jr. et al. *Neointimal thickening after severe coronary artery injury is limited by short-term administration of a factor Xa inhibitor. Results in a porcine model.* Circulation 1996, 93: 1542-8.

110. Erickson, B.R., Topol, E.J. *Direct thrombin inhibitors in arterial thrombosis.* In: New Therapeutic Agents in Thrombosis and Thrombolysis. Sasahara, A.A., Loscalzo, J. (Eds.). Marcel Dekker, Inc.: New York 1997, 169-89.

111. Crowther, M.A., Ginsberg, J.S. *Direct thrombin inhibitors in venous thromboembolism.* In: New Therapeutic Agents in Thrombosis and Thrombolysis. Sasahara, A.A., Loscalzo, J. (Eds.). Marcel Dekker, Inc.: New York 1997, 159-68.

112. Hermann, J.P.R., Kutryk, M.J.B., Serruys, P.W. *Clinical trials of direct thrombin inhibitors during invasive procedures.* Thromb Haemost 1997, 78: 367-76.

113. Verstraete, M. *Direct thrombin inhibitors: Appraisal of the antithrombotic/hemorrhagic balance.* Thromb Haemost 1997, 78: 357-63.

114. White, H.D. *Clinical trials of direct thrombin inhibitors in acute ischemic syndromes.* Thromb Haemost 1997, 78: 364-6.

115. Vermeer, F., Vahanian, A., Fels, P.W. et al. *Intravenous argatroban versus heparin as co-medication to alteplase in the treatment of acute myocardial infarction: Preliminary results of the ARGAMI pilot study.* J Am Coll Cardiol 1997, 29: 185-6.

116. Lewis, B.E., Grassmann, E.D., Wrona, L., Rangel, Y. *Novastan® anticoagulation during renal stent implant in a patient with heparin-induced thrombocytopenia.* Blood Coag Fibrinol 1997, 8: 54-8.

117. Kaiser, B. *A survey of anti-Xa-based drugs: Potential use in cardiovascular indications.* In: New Anti-coagulants for the Cardiovascular Patient. Pifarré, R. (Ed.). Hanley & Belfus, Inc.: Philadelphia 1997, 431-41.

118. Wong, P.C., Crain, E.J., Nguan, O., Watson, C.A., Racanelli, A. *Antithrombotic actions of selective inhibitors of blood coagulation factor Xa in rat models of thrombosis.* Thromb Res 1996, 83: 117-26.

119. Yamashita, T., Tsuji, T., Matsuoka, A., Giddings, J.C., Yamamoto, J. *The antithrombotic effect of synthetic low molecular weight human factor Xa inhibitor, DX-9065a, on He-Ne laser-induced thrombosis in rat mesenteric microvessels.* Thromb Res 1997, 85: 45-51.