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Review

Direct thrombin inhibitors – a survey of recent developments

A. Schwienhorst

Department of Molecular Genetics and Preparative Molecular Biology, Institute for Microbiology and Genetics, Grisebachstraße 8, 37077 Goettingen (Germany), Fax: +49 551 393805, e-mail: aschwie1@gwdg.de

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Abstract. Thrombin is a plasma serine protease that plays a key role in coagulation and hemostasis but also in thromboembolic diseases. Direct thrombin inhibitors could, therefore, be beneficial for future anticoagulant therapy in the prophylaxis of venous and arterial thrombosis as well as myocardial infarction. However, development of direct thrombin inhibitors has brought researchers more heartache than success. The most recent setback came this year when AstraZeneca withdrew Ximelagatran, the first orally bioavailable direct thrombin inhibitor that had received regulatory approval (France, 2003), after reports of serious hepatoxicity in a fraction of patients. This review describes the status of direct thrombin inhibitors, focusing on drug candidates that are at present in clinical trials. In addition, some more recent research strategies in the design of novel direct thrombin inhibitors are discussed, which may very well contribute to future developments of potent anticoagulants.

Keywords. Direct thrombin inhibitor, hirudin, anticoagulant therapy, thromboembolic disease, drug candidate, oral bioavailability, neutral P1 moiety.

Introduction

Thromboembolic diseases are known as the major cause of mortality and morbidity worldwide [1]. At present, treatment to a large extent relies on indirect anticoagulants, the standard therapy being to administer parenteral anticoagulants such as heparins. The anticoagulative response to heparins, however, varies between patients, mainly due to the plasma protein-binding properties of heparins [2]. Heparins, i.e. heparin/antithrombin complexes, are not very active against fibrin-bound thrombin [3]. Furthermore, heparins show reduced activity in the vicinity of platelet-rich thrombi, due to high concentrations of platelet factor 4 [4]. In addition, formation of platelet factor 4-heparin complexes in some cases exposes neoantigens on platelet factor 4, which then trigger the formation of antibodies that cause heparin-induced thrombocytopenia (HIT) [5]. With the development of low-molecular-weight heparins (LMWHs), which show less plasma protein binding, undesired side effects could in general be moderated, although a risk of bleeding complications has recently been reported for the heparin derivative Fondaparinux [6]. Application of vitamin K antagonists, such as the coumarin derivative warfarin, has represented the only approved approach to oral anticoagulation for more than 50 years, although the agents have significant shortcomings that result mainly from their nonspecific mechanisms of action and rather variable pharmacodynamics. Patients require frequent laboratory monitoring due to the narrow therapeutic window of the drugs, i.e. the risk of either thrombotic complications or hemorrhage [7]. The need for coagulation monitoring also arises from multiple drug-drug and drug-diet interactions, and further complications arise from the slow onset of action [7]. As a consequence, drugs like warfarin are often co-administered with parenteral anticoagulants, at least at the beginning of therapy. Since the aforementioned indirect anticoagulants obviously have several drawbacks, as early as in the mid-1980s, the goal was set to develop new and preferentially oral anticoagulants that could, in particular, replace vitamin K antagonists.

Thrombin as a target for pharmacological intervention

In principle, many targets in the coagulation cascade may be suitable for pharmacological intervention. Currently, anticoagulants in more advanced stages of development focus on a few targets, such as tissue factor-bound factors VIIa, IXa, Xa, VIIIa, Va and thrombin [8]. The latter plays a central role in blood coagulation and therefore became one of the most popular targets for the discovery of novel antithrombotic agents [9, 10]. Procoagulant activities of thrombin include the conversion of fibrinogen into clotable fibrin, activation of the fibrin cross-linker factor XIII, activation of cofactors V and VIII and the down-regulation of fibrinolysis by generating thrombin-activatable fibrinolysis inhibitor [11, 12]. In addition, thrombin also mediates platelet activation and aggregation by acting on protease-activated receptors [13, 14]. However, human thrombin also executes anticoagulant activities. In the presence of thrombomodulin and most efficiently at low sodium concentrations, it generates protein C [15, 16]. This protease together with protein S inactivates factors Va and VIIIa and thereby down-regulates blood coagulation [17, 18]. Because of its central role in coagulation and hemostasis, thrombin is considered to be an ideal target for anticoagulant therapy, and tremendous efforts have been undertaken particularly during the last two decades to develop preferentially small-molecule direct thrombin inhibitors [reviewed in refs. 19, 20].

Thrombin's accessible surface for inhibitor binding

Thrombin is a trypsin-like serine protease that catalyzes the conversion of fibrinogen to fibrin as well as the processing of a number of other targets. Nearly exclusively, it cleaves peptide bonds after arginine residues (Table 1) [21]. Human thrombin comprises an A chain of 36 amino acids and a B chain of 259 amino acids, linked by a disulfide bridge. Like other serine proteases, it contains the catalytic triad Asp102, His57 and Ser195. As a peculiarity, thrombin shows an insertion loop (Tyr60A, Pro60B, Pro60C, Trp60D) which is not present in other serine proteases and which limits the access of peptidic substrates as well as inhibitors to the active-site region. Grossly, the active-site region comprises three distinct pockets. The specificity pocket (S1) of thrombin, as well as in trypsin, has an aspartic acid (Asp189) at the bottom which determines the preference of the enzymes for substrates with a basic P1 residue like arginine. As a result of the presence of the insertion loop, the neighboring S2 pocket usually accomodates relatively small and hydrophobic substrate residues such as proline. The distal pocket or aryl-binding site (S3/4) is separated from the S2 site by Leu99 and is further lined with Ile174 and Tryp215. Not surprisingly, it can harbor hydrophobic and aromatic moieties. For recognition of natural substrates such as fibrinogen or thrombin receptors, regions distant from the active-site are also important [21]. In particular, exosite I, which is located 20 Å away from the active-site, and the heparin exosite, both of which are composed of several basic lysine and arginine side chains, contribute significantly to the specificity and affinity of substrate binding [21, 22]. Whereas interaction of natural peptidic thrombin inhibitors such as hirudin [23, 24], bothrojaracin [25], triabin [26], rhodniin [27], ornithodorin [28] and dipetalogastin [29] also includes exosite binding, designed small-molecule inhibitors in general interact exclusively with the residues of the active-site pockets of thrombin. For example, many low-molecular-weight inhibitors, such as argatroban **10** (Fig. 1) or melagatran **13** (Fig. 2), contain basic moieties to mimic the binding of P1 arginine in natural substrates (Table 1) to Asp189 in the S1 pocket.

Major constraints in the design of direct thrombin inhibitors

Therapeutically useful thrombin inhibitors have to meet several needs beyond showing low inhibition constants

Table 1. Thrombin cleavage sites in peptidic substrates.

Substrate	Cleavage site						
	P_3	P_2	P_1	⇓	P_1'	P_2'	P_3'
Fibrinogen α chain	G	V	R		G	P	R
Fibrinogen β chain	S	A	R		G	H	R
Thrombin receptor-like PAR1	D	P	R		S	F	L
Thrombin receptor-like PAR3	P	I	K		T	F	R
Thrombin receptor-like PAR4	A	P	R		G	Y	P
Factor II	T	P	R		S	E	G
Factor II	N	P	R		T	F	G
Factor V	G	I	R		S	F	R
Factor V	S	P	R		T	F	A
Factor VII	Q	G	R		I	V	G
Factor VIII	Q	I	R		S	V	A
Factor VIII	E	P	R		S	F	S
Factor XI	K	P	R		I	V	G
Faktor XIII	V	P	R		G	V	N
Protein C	D	P	R		L	I	D
Thrombin-activatable fibrinolysis inhibitor	S	P	R		A	S	A
Fluorogenic substrate consensus [1]	X	P	R		–	–	–

[1] Tetrapeptides containing the fluorogenic leaving group 7-amino-4-carbamoylmethylcoumarin [207]. X can be any amino acid.

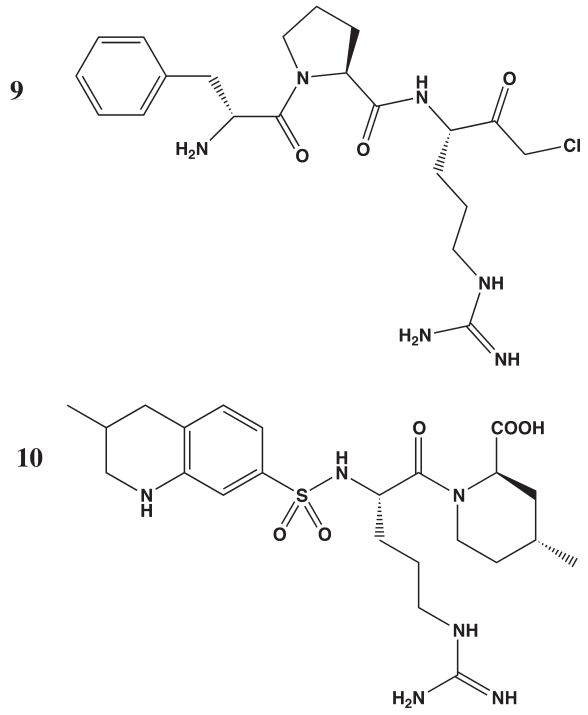


Figure 1. Early low-molecular-weight direct thrombin inhibitors PPACK (**9**) and Argatroban (**10**).

for the target enzyme thrombin. However, low inhibition constants do not always lead to a potent anticoagulant effect. In particular, significant plasma protein binding of inhibitors, originating e.g. from the hydrophobicity of the molecules, limits the effective inhibitor concentration and thus impairs the anticoagulant effect [30–33]. Plasma protein binding, however, is also an important feature that determines the pharmacokinetics of a given compound, e.g. its distribution and elimination. Fast elimination with predominant hepatobiliary excretion has been a problem for many thrombin inhibitors [34]. Molecular properties affecting plasma clearance and biliary excretion have been studied in a series of NAPAP analogs. Here, elimination could be significantly lowered by introducing additional ionizable moieties [35, 36]. Dibasic compounds were eliminated particularly slowly. Similar results were reported recently for another thrombin inhibitor scaffold [37]. In agreement with these findings, relatively short half-lives were observed for thrombin inhibitors with a nonbasic group in the P1 position [38]. It is mandatory that inhibitors exhibit a high degree of selectivity, and enzymes of the fibrinolytic system should not be inhibited [39–41]. Last but not least, the ideal thrombin inhibitor should be chemically and metabolically stable and nontoxic [42, 43].

Among other important factors, one prerequisite for effective oral bioavailability is effective intestinal absorption. Among the most important properties of a compound to favor its enteral absorption are sufficient lipophilicity, low molecular weight and a small number of hydrogen bond donors and acceptors [44]. In particular, compounds with ionizable functionality are less well absorbed than neutral compounds [45]. In addition to these properties, usually subsumed under 'Lipinski's rule of 5', other properties have been discussed. These include molecular flexibility [46, 47], water complexation by amide bonds [48, 49], as found in peptidomimetic compounds, and a polar surface area [47, 50]. The ideal thrombin inhibitor has to meet conflicting demands, e.g. a certain degree of polarity to prevent insolubility, plasma protein binding and fast elimination together with a certain degree of lipophilicity to favor enteral absorption. Thus, a well-balanced compromise has to be found in the optimization of the different properties of thrombin inhibitors, and the design of therapeutically suitable thrombin inhibitors is, consequently, a multiparameter problem. Concerning this point, promising news recently came from a novel computer-assisted optimization approach that is able to manage this task of multiparameter thrombin inhibitor optimization efficiently [37].

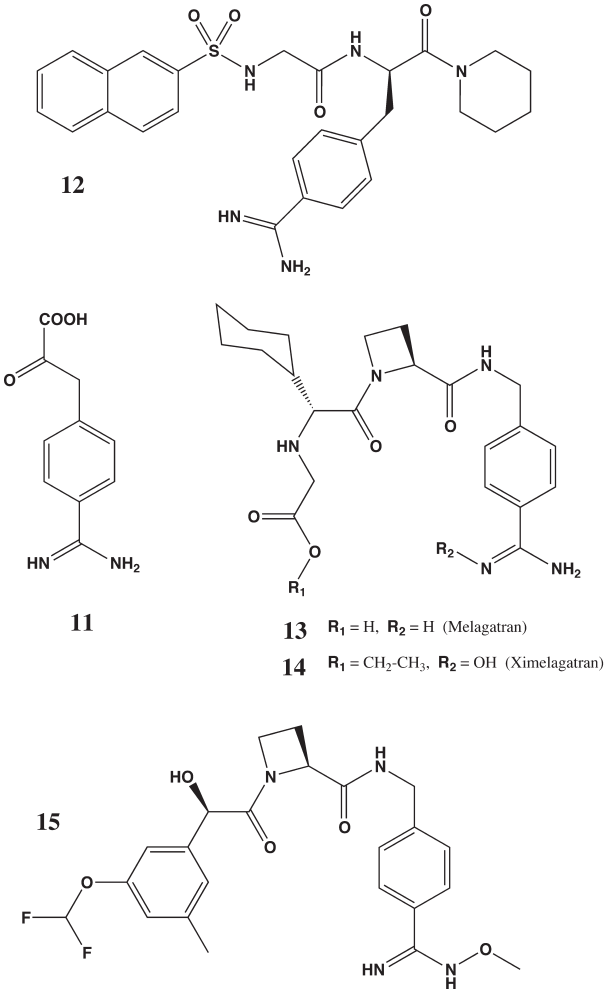


Figure 2. Benzamidine-based low-molecular-weight inhibitors.

Early direct thrombin inhibitors

The use of direct thrombin inhibitors in the form of leeches stretches back to very ancient times. Their first use in medicine was recorded by Nicander of Colophon (200–130 BC) particularly in his hexameter poems *Alexipharmaca*, on poisons and their antidotes, and *Theriaca*, which deals with the nature of venomous animals and the wounds they inflict [51]. However, it was not until 1884 that Haycraft identified the anticoagulant in the saliva of medicinal leech, which was termed hirudin. Isolation of pure hirudin variants by Markwardt and coworkers around 1955 [23, 52, 53] was followed by determination of the primary structure [53–57]. Cloning and sequencing of the hirudin gene [58] allowed large-scale production of recombinant hirudin, e.g. in *Escherichia coli* [59] and *Saccharomyces cerevisiae* [60]. Whereas natural hirudins are single-chain polypeptides of approximately 65–66 amino acids containing a sulfated tyrosine, recombinant forms lack the sulfate group on tyrosine 63 [61], and therefore bind less tightly to thrombin. The three-dimensional structure of hirudin in complex with thrombin [24, 62] revealed that hirudin is a bivalent inhibitor. The compact amino-terminal core domain blocks the active-site of thrombin but does not bind to the S1 pocket, whereas the extended carboxy-terminal domain binds to the fibrinogen exosite of the enzyme. Mutations particularly in the carboxy-terminal domain as in protease-resistant variants generated by directed evolution [63] significantly reduced inhibitory activity. Although many other, highly efficient thrombin inhibitors have been discovered in blood-sucking animals, only two recombinant forms of hirudin, lepirudin **1** (Refludan) and desirudin **2** (Revasc) have entered the clinic so far (Fig. 3). Desirudin **2** (IPRIVASC) was approved by the FDA in 2003 for prophylaxis of deep-vein thrombosis in patients undergoing voluntary hip replacement surgery. Lepirudin **1** (Refludan) was approved by the FDA in 1998 for use in patients with HIT and related thrombosis. However, due to bleeding problems [64] and a short half-life ($t_{1/2}$: 60–80 min after intravenous administration), the clinical use of recombinant hirudins is limited. Furthermore, antihirudin antibodies have been determined in a high percentage of patients receiving lepirudin, and fatal anaphylaxis has been described for patients within 3 months after lepirudin treatment [65, 66].

Based on the hirudin principle of bivalent binding to thrombin, a number of synthetic thrombin inhibitors have been designed (Fig. 3). In hirulog-1 **3** (bivalirudin, Angiomax; [67]) and the peptidic inhibitor P53 **4** [68], the dPhe-Pro-Arg sequence that mimics the specific binding of human fibrinopeptide A is fused to a large part of the hirudin fibrinogen exosite binding domain by means of a small peptidic linker. Both hirulog-1 **3** (K_i = 2.3 nM) and P53 **4** (K_i = 2.8 nM) are strong inhibitors of human α-thrombin. For hirulog-1, a rather short plasma half-life was observed ($t_{1/2}$: 25 min). Much weaker binding was observed with bivalent peptides where the dPhe-Pro-Arg motif was replaced by sequences of thrombin-activated receptors [69–71] or part of the natural sequence of human fibrinopeptide A [72]. All these constructs, however, contained a scissile bond after the P1 arginine residue that is slowly cleaved by thrombin. Later, several groups developed bivalent thrombin inhibitors with improvements in proteolytic stability, e.g. against thrombin cleavage, enhanced inhibitory efficacy, avoidance of nonnatural

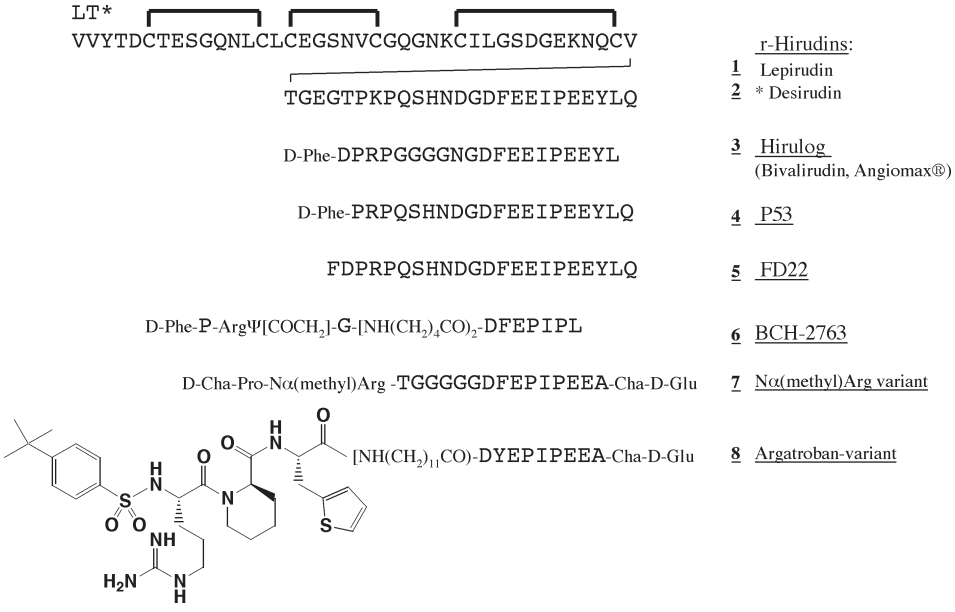


Figure 3. Bivalent direct thrombin inhibitors based on sequences of the leech anticoagulant hirudin.

amino acids and size reduction. Stability against thrombin cleavage was achieved by introducing proteolytic-resistant functions at the P1 position such as β-homo amino acids ArgΨ[CH_2CONH], as in **6**, and reduced bond analog ArgΨ[CH_2NH] [73] or Nα(methyl)arginine, as like in **7** [74]. More recently, the incorporation of optimized, substrate-like tetrapaptides consisting exclusively of natural amino acids, as in **5**, was also reported as a successful strategy to confer resistance against thrombin cleavage [75]. More potent inhibitors were obtained by replacing the active-site-binding segment by small-molecule inhibitor segments derived from the nonelectrophilic inhibitors argatroban **10** (to yield **8**) [76] or NAPAP **12** [77], and from electrophilic inhibitors such as boronic acids [78], arginyl methyl ketones [79, 80] and α-keto amides [81]. Furthermore, a series of bivalent thrombin inhibitors termed hirunorms was generated that contain a peptidic module that blocks the active-site in a non-substrate mode [82, 83]. Other variations of the initially developed hirulogs include modifications in the linker [84, 85] and the fibrinogen exosite-binding region [86, 87] as well as size reduction of the hirudin-derived part, as in BCH-2763 [88]. More recently, a chimera of hirudin and dipetalogastin II, a potent peptidic thrombin inhibitor from the bug *Dipetalogaster maximus* was described which exhibited an inhibition equilibrium constant (K_i) of 45 fM [89] which is close to that of natural hirudin. Nevertheless, despite the fact that major improvements have been made through modifications of the original hirulogs, bivalirudin (hirulog-1, Angiomax) is still the only hirulog that has reached the market, being approved by the FDA in 2000 as an anticoagulant in patients with severe chest pain (unstable angina) undergoing a procedure to open blocked arteries in the heart (percutaneous transluminal coronary angioplasty, PTCA).

Early findings that fibrinopeptide A and, in particular, tripeptides FVR and FPR inhibit thrombin cleavage of fibrinogen [90, 91] gave rise to the idea that such small structures could be used as antithrombotic drugs. Early synthetic compounds included the covalent thrombin inhibitors PPACK **9** [92] and noncovalent inhibitor argatroban **10** [93], both of which contain an arginine as a P1 residue (Fig. 1). The latter was the first low-molecular-weight direct thrombin inhibitor that reached the market. It was approved by the FDA in 2000 for anticoagulation in the prophylaxis or treatment of thrombosis in patients with HIT and in 2002 for patients at risk of HIT while undergoing percutaneous coronary intervention. Argatroban **10** is a rather selective reversible inhibitor (K_i = 38 nM for human thrombin) with only minor inhibitory activity against other serine proteases such as trypsin, factor Xa and kallikrein [93]. Compared to the hirudins, argatroban **10** is advantageous since it does not induce antibodies [94] and is capable of neutralizing clot-bound thrombin [95]. It has a short duration of action ($t_{1/2}$: 40 min). Despite its low molecular weight, argatroban **10** has to be applied parenterally due to the presence of the highly basic guanidine moiety which prevents absorption from the gastrointestinal tract. Furthermore, clinical studies with non-HIT patients have shown that argatroban **10** was not really advantageous compared to heparin with respect to infarct-related artery and bleeding complications [96, 97].

The case of ximelagatran

All of the aforementioned approved direct thrombin inhibitors have so far only established niche applications in clinical practice, mainly because of the need for parenteral administration and frequent laboratory monitoring. Therefore, much attention was attracted by AstraZeneca's ximelagatran **14** (Exanta), the first oral direct thrombin inhibitor that received regulatory approval (France, 2003) for the prevention of venous thromboembolic events in major orthopedic (hip or knee replacement) surgery. In 2004, it was launched in Germany as its first market worldwide. Ximelagatran **14** (Fig. 3) is a prodrug derivative of melagatran **13**. Melagatran **13** belongs to the group of peptidomimetic thrombin inhibitors that mimic the D-Phe-Pro-Arg sequence [43]. It contains a benzamidine side chain as a P1 moiety replacing the arginine. Benzamidines such as 4-amindinobenzylamine [98] and 4-amidinopyruvic acid **11** [99] have long been known to be potent inhibitors of serine proteases and had already been introduced into peptidomimetic thrombin inhibitors such as NAPAP **12** [100].

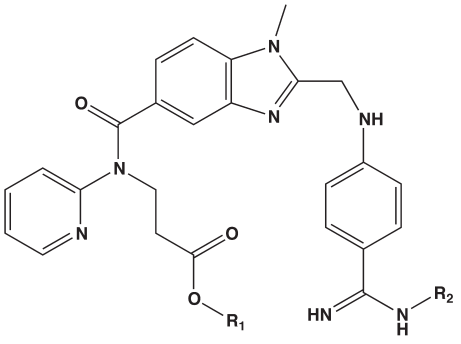
In melagatran **13**, proline as the P2 moiety is mimicked by azetidine 2-carboxylic acid whereas D-cyclohexylglycine (D-Cha) replaces D-Phe in the P3 position. This binding mode has been confirmed by crystallographic studies [101]. Melagatran is a potent active-site inhibitor of thrombin and trypsin, with K_i values of 3 and 4 nM, respectively [37, 102]. However, it has sufficient selectivity towards fibrinolytic enzymes [102]. Melagatran inhibits both free and clot-bound thrombin with reproducible pharmacokinetic properties, low plasma protein binding and mainly renal clearance [103, 104]. Melagatran **13** shows a plasma half-life of 2–3 h. Animal models and clinical trials suggest that melagatran **13** has a wider therapeutic window than warfarin [105, 106]. Unfortunately, melagatran **13** has only low bioavailability (5.8%) after oral administration due to poor adsorption [104]. To overcome this problem, the double prodrug ximelagatran **14** (H376/95, Exanta) was developed. Actually, the carboxylic group is esterified and the basic amidino group is converted into an amidoxime moiety to increase lipophilicity [104]. In humans, ximelegatran is well absorbed in the small intestine with a bioavailability in the range of 20% [107] and low inter-subject variation [106]. Because it is not metabolized by the hepatic cytochrome

P450 system, there is a low incidence of drug-drug interactions and no relevant food dependence [106]. Efficacy, e.g. with fixed dosing every 12 h, was demonstrated in a number of clinical trials [for reviews see refs. 106, 108, 109]. The safety profile with respect to bleeding risk appears favorable for ximelagatran **14** relative to warfarin as monotherapy [108]. The conclusion from two large clinical trials (SPORTIF III and V), was that ximelagatran was as effective as warfarin in preventing stroke and systemic embolism, because a previously set noninferiority criterion was met [110, 111]. Reexamination of the data with rather conservative interpretive criteria, however, revealed a number of deficiencies, e.g. inappropriateness of the analytic method used and an unreasonably generous margin that was possibly biased towards noninferiority [112, 113]. In addition, a major concern that became apparent was the high incidence of liver enzyme elevation observed in patients undergoing prolonged therapy. In particular, 7.9% of all patients receiving ximelagatran **14** for more than 35 days showed an increase in alanine aminotransferase more than threefold above the upper limit of normal. The combination of this elevation together with a total bilirubin level of more than twofold above the normal upper limit occurred in 37/6948 patients. Despite nine deaths in this group, it was believed that none of these cases was directly related to hepatic failure caused by ximelagatran [108, 114]. However, reexamination of the clinical data appeared to undermine the noninferiority claim for ximelagatran and led the United States FDA Cardiovascular and Renal Drugs Advisory Committee to recommend against approval of ximelagatran **14** in 2004. As a result, AstraZeneca withdrew ximelagatran **14** and stopped all clinical trials in February 2006 [115]. AstraZeneca will instead focus on the development of the follow-up compound AZD0837 **15** (Fig. 1) which is already in phase II studies. However, no detailed information is available so far.

Direct thrombin inhibitors in clinical trials

With the leading horse of oral direct thrombin inhibitors dropping out of the race, Boehringer Ingelheim's BIBR-1048 **17** (dabigatran etexilate; RENDIX; Fig. 4) is presumably next in line, as it is currently in phase III trials for venous thrombosis and atrial fibrillation [116, 117]. BIBR-1048 **17** is a prodrug that is rapidly converted to its active form BIBR-953 ZW **16** (dabigatran). It was developed as a constrained derivative of NAPAP **12** mainly by replacing the central glycine residue by a trisubstituted benzimidazole [118]. BIBR-953 ZW **16** is a potent thrombin inhibitor (K_i = 4.5 nM) with only moderate selectivity toward trypsin (K_i = 50.3 nM) [119, 120]. Interestingly, binding energy mainly results from hydrophobic interactions, in addition to the salt bridge to Asp189. BIBR-1048 **17** is orally active and exhibits a rather slow clearance ($t_{1/2}$: 12 h). A multicenter, dose-escalating study with 314 patients receiving oral doses of BIBR-1048 **17** twice daily revealed no major bleeding events [116]. Only in two patients receiving the highest dose (300 mg) was bleeding from multiple sites observed associated with decreased renal clearance. A second multicenter study on the prevention of venous thromboembolism after orthopedic surgery revealed that venous thromboembolism was significantly lower in patients receiving BIBR-1048 **17** (150 or 225 mg twice daily or 300 mg once daily) compared with patients receiving enoxaparin [117]. In January 2006, Boehringer Ingelheim reported the launch of the biggest thromboembolic disease clinical trial program (RE-VOLUTION) ever. Target indications include stroke prevention in atrial fibrillation, prevention of deep-vein thrombosis after orthpaedic surgery, acute deep-vein thrombosis treatment and its secondary prevention.

On the basis of the argatroban **10** structure, Sanofi-Synthélabo developed the oral competitive thrombin inhibitor SSR182289A **18** (Fig. 4) which does not require administration in the form of a prodrug [121, 122]. This was mainly achieved by incorporating a weakly basic 3-amino-pyridine as the P1 residue. SSR182289A **18** inhibits human thrombin (K_i = 31 nM) and exhibits a



16 R_1 = H, R_2 = H (BIBR-953 ZW)

17 R_1 = CH_2-CH_3, R_2 = COO-$(CH_2)_5$-CH_3 (BIBR-1048)

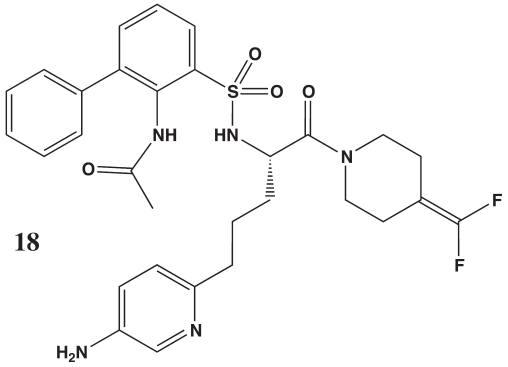


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Figure 4. Direct thrombin inhibitors BIBR-953 ZW(**16**), BIBR-1048 (**17**) and SSR182289A (**18**).

good selectivity toward trypsin (K_i = 54 μM) and other human proteases [121]. Data from the rat model suggest a prolonged duration of action at least similar to that of melagatran **13** [122, 123]. Furthermore, a recent study on a rabbit thrombolysis model shows that SSR182289A **18** may enhance thrombolysis induced by either recombinant tissue plasminogen activator or streptokinase [124]. SSR182289A **18** (Fig. 4) has been reported to be in phase IIa clinical studies for general thrombosis.

Based on the tripeptidic covalent thrombin inhibitor DUP 714 **19** [125] which shows an inhibition equilibrium constant (K_i) of 41 pM, derivatives were synthesized that exhibited greater selectivity [126] and improved oral bioavailability [127] eventually leading to TRI50c **20** (Fig. 5) where the basic arginine side chain was eliminated [128]. TRI50c **20** is a potent inhibitor of thrombin (K_i = 9 nM) with high selectivity toward trypsin (K_i = 1000 nM) and even more pronounced toward other human serine proteases. It shows high efficacy in animal models of venous and arterial thrombosis with a minimal risk of bleeding complications. TRI50c **20** is the active principle in two compounds, TGN 255 (parenteral formulation), and TGN 167 (oral formulation), that are currently being developed by Trigen Holdings AG, a biotechnology company with operations in both London and Munich. TGN 255 shows fast clearance in humans with no adverse clinical events

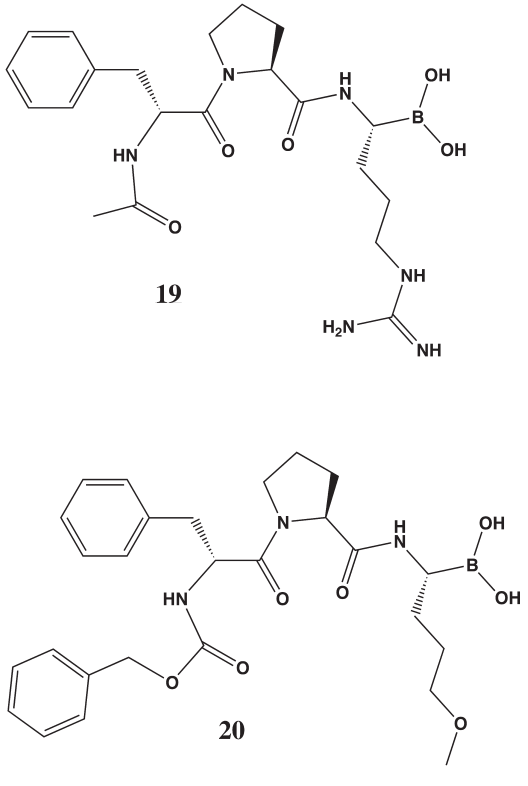

Figure 5. Direct thrombin inhibitors DUP 714 (**19**) and Trigen's TRI50c (**20**).

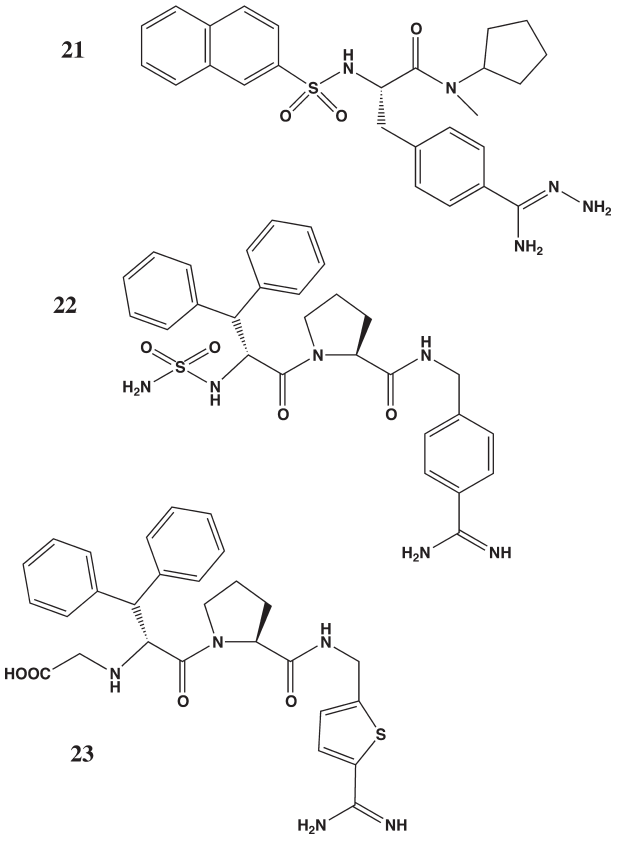

Figure 6. LGLS' direct thrombin inhibitors LB-30057 (**21**), LB-30812 (**22**) and LB-30870 (**23**).

such as hemorrhage or liver injury [129]. It recently completed successfully a phase II clinical study for anticoagulation in hemodialysis. A phase III trial in hemodialysis patients is due to start in 2006. TGN 167, Trigen's oral thrombin inhibitor is currently in phase I clinical studies. Furthermore, Trigen has recently reported the development of a product-specific antidote (TGN 212) blocking TGN 167 and allowing neutralization of the active anticoagulant principle in TGN 167.

Previously, LG Life Sciences had developed the tripeptide-like inhibitor LB-30057 **21** following the strategy to include the less basic arginine surrogate as a P1 moiety (Fig. 6). The inhibitor showed an inhibition equilibrium constant (K_i) of 0.38 nM for thrombin and proved to be selective and orally bioavailable. However, further development ceased in 2003 due to its low efficiency and short duration of action in phase I clinical trials [130]. Following the line of benzamidrazone inhibitors eventually lead to one of the most potent synthetic thrombin inhibitors ever, LB-30812 **22** (K_i = 3 pM), which is also highly selective and very effective in rat and rabbit thrombosis models. However, oral bioavailability in rats, dogs and monkeys was only moderate. Further derivatization eventually lead to LB-30870 **23** (Fig. 6), which combines a thienylamidine as a less basic P1 moiety and a carboxylic

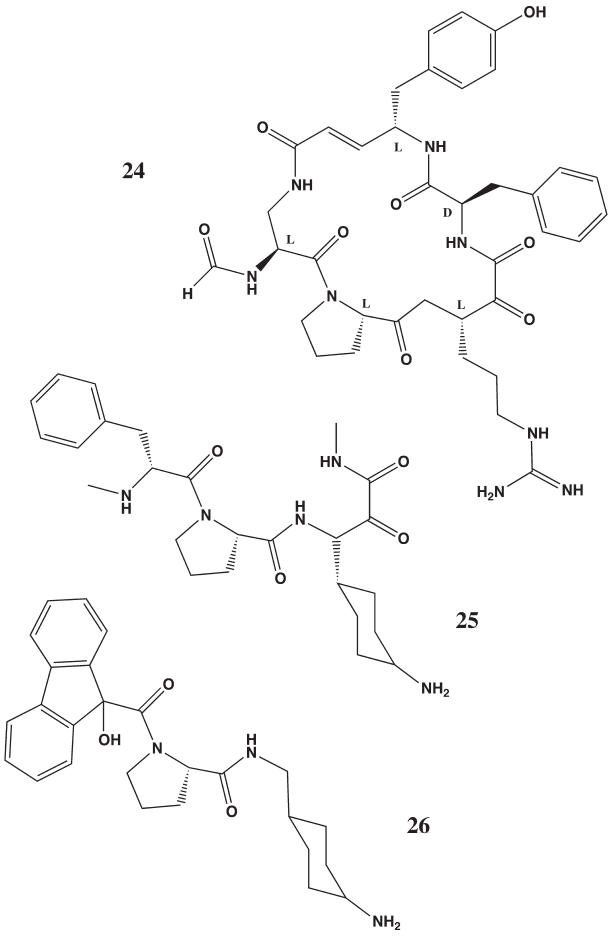

Figure 7. Direct thrombin inhibitors cyclotheonamide (**24**), L-370,518 (**25**) and L-372,460 (**26**).

acid moiety. Interestingly, nearly simultaneously, BASF developed inhibitors sharing the same motifs [131, 132]. LB-30870 **23** is a very potent thrombin inhibitor (K_i = 15 pM) with a moderate selectivity towards trypsin (K_i = 300 pM). It exhibits good oral bioavailability in rats (F = 43%), dogs (F = 42%) and monkeys (F = 15%) with no significant toxic sign (mice, rats, monkeys). It proved to be twentyfold more efficacious than low-molecular-weight heparin (enoxaparin) in a rabbit venous thrombosis model. Initial data from phase I clinical trials show that LG Life Sciences' LB-30870 **23** is well absorbed in humans and highly efficacious, with no significant bleeding even at high doses [133, 134].

Several years ago, Merck started a thrombin inhibitor program based on the structure of cyclotheonamide A **24** [135], a macrocyclic peptide from *Theonella* sp. (Fig. 7). Macrocyclic peptides still attract some attention in thrombin inhibitor research [136]. Cyclotheonamide A **24** showed only modest thrombin inhibitor potency (K_i = 0.18 μM) but was a potent inhibitor of trypsin (K_i = 0.023 μM) and streptokinase (K_i = 0.035 μM). To improve selectivity towards thrombin, derivatives such as L-370,518 **25** (K_i = 0.5 nM for thrombin; K_i = 1.15 μM for trypsin) and L-372,460 **26** (K_i = 1.5 nM for thrombin; K_i = 0.86 μM for trypsin) were developed [137] (Fig. 7). Replacement of the P2-P3 module by aminopyridinone and introduction of a 5-linked 2-amino-6-methylpyridine generated L-374,087 **27** (K_i = 0.5 and 3200 nM for thrombin and trypsin, respectively [138]; Fig. 8). L-374,087 **27** was efficacious in a rat model of arterial thrombosis and showed oral bioavailability in dogs and monkeys [138, 139]. However, metabolic stability was a problem [140–143]. Introduction of an electron-withdrawing group into the central pyridine ring, i.e. replacement of the P2-P3 module by an aminopyrazinone moiety, lead to the more metabolically stable L-375,378 **28** (Fig. 8), with K_i values of 0.8 and 1800 nM against thrombin and trypsin, respectively, and excellent pharmacokinetic properties (F = 92%; $t_{1/2}$: 185 min in dogs) [144]. In an effort to improve metabolic stability and reduce further the basicity of the P1 moiety, a fluoropyridyl derivative **29** was developed [145]. Compound **29**, however, showed only moderate aqueous solubility. Further development lead to a pyridine N-oxide derivative **30** (Fig. 8) with a simple

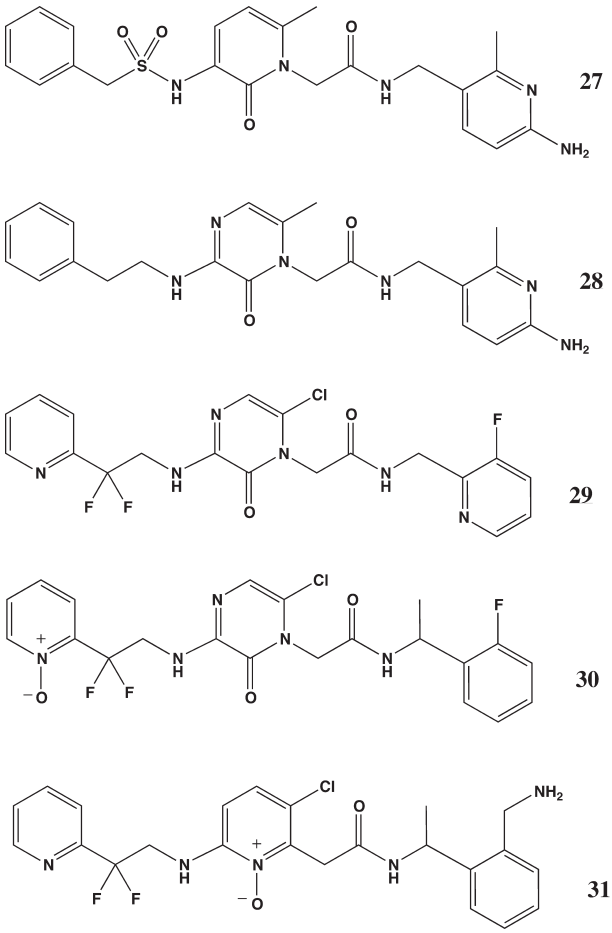

Figure 8. Direct thrombin inhibitors L-374,087 (**27**), L-375,378 (**28**), fluoropyridyl- (**29**) and N-oxide derivatives (**30** and **31**).

fluoro-substituted benzylamide P1 moiety [146] which was reported to combine good thrombin inhibitory activity (K_i = 2.3 nM), improved solubility and good oral bioavailability and half-life ($t_{1/2}$: 4.9 h in dogs). Two candidates of these series of compounds have been reported to have advanced into phase I clinical trials [147, 148].

More recently, Merck reported on a different approach to prevent oxidative metabolism, i.e. the introduction of pyridine N-oxide in the P2 position [149]. From this series, compound **31** (Fig. 8) showed a good thrombin inhibitory potency (K_i = 3.2 nM) and an increased plasma half-life in dogs ($t_{1/2}$: 4.5 h). In a recent report on a new line of development, researchers again took compound L-372,460 **26** as a starting point. From the new series, one compound, **32** (Fig. 9), combines high potency (K_i = 0.40 nM), excellent pharmacokinetic properties (F = 55%; $t_{1/2}$: 14 h in dogs) and complete efficacy in an *in vivo* rat thrombosis model [150]. Chances for further development may also originate from a new oxazolpyridine series [151]. The dual thrombin/factor Xa inhibitor **33** (Fig. 9) showed good thrombin and factor Xa inhibitory activity (K_i = 0.04 nM and 3.9 nM, respectively), a rather long half-life ($t_{1/2}$: 4.2 h in dogs) and complete efficacy in an *in vivo* rat thrombosis model, but unfortunately exhibited only low levels of oral bioavailability.

Following earlier work [152, 153], an interesting new class of orally bioavailable thrombin inhibitors based on oxyguanidines as guanidine bioisosteres has recently been developed by 3-Dimensional Pharmaceutics [154]. As an example of this early series, compound **34** (Fig. 10) showed good thrombin inhibitory activity (K_i = 11.9 nM), good selectivity and excellent pharmacokinetic properties (F = 95%; $t_{1/2}$: 7.3 h in dogs). Further derivatization revealed compounds like **35** (Fig. 10) which showed improvements in thrombin inhibitory potency (K_i = 4 nM) while retaining very good pharmacokinetic properties (F = 73%; $t_{1/2}$: 4.4 h in dogs). Metabolic stability (>98%) and absorption characteristics as judged by permeability data from Caco-2 assays appeared to be favorable. However, plasma protein binding was reported to limit antithrombotic efficacy *in vivo* [155]. In 2000, a phase I trial was reported for a nonspecified clinical candidate (3DP-4815), which had been found during the aforementioned structure-based drug discovery programs [156] but since then no further information has been published.

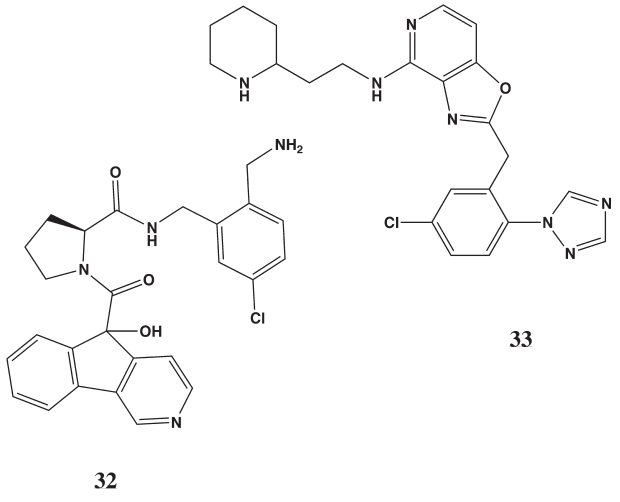

Figure 9. Merck's recent direct thrombin inhibitors (**32**) and (**33**).

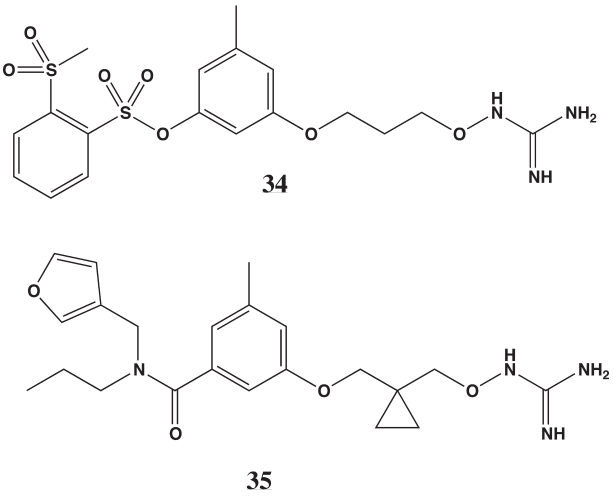

Figure 10. 3D Pharmaceuticals' direct thrombin inhibitors (**34**) and (**35**).

Aptamers are rather short, single-stranded oligonucleotides that adopt specific, stable conformations and bind tightly to very specific target molecules. Already shortly after the invention of the SELEX technology [157, 158] for the generation of aptamers by *in vitro* selection Gilead Sciences Inc. developed the 15-nucleotide DNA-based antithrombin aptamer (5′-GGTTGGTGTGGTTGG-3′) that selectively binds thrombin with moderate affinity ($K_d = 10^{-7}$ M) [159]. Crystallographic structure determination revealed that the aptamer folds into a structure with two G-quartets and binds the fibrinogen exosite of one thrombin molecule and the heparin exosite of a second thrombin molecule [160]. To take advantage of its rapid clearance ($t_{1/2}$: 2 min), the antithrombin aptamer was developed for use in surgical indications where regional anticoagulation with rapid onset and reversal of action is required. When administered by constant infusion, the DNA aptamer successfully replaced heparin in a canine cardiopulmonary bypass model [161, 162]. Furthermore, the aptamer was able to inhibit clot-bound thrombin and platelet thrombus formation in an ex vivo whole-artery angioplasty model [162]. Although Gilead has ceased the development of aptamers, Archemix Corporation and Nuvelo Inc. recently entered into a collaboration to continue its development for coronary artery bypass graft surgery, percutaneous coronary intervention and other acute an-

ticoagulant applications. In September 2005, Archemix and Nuvelo released preliminary results from a phase I trial evaluating 15mer-DNA aptamer ARC183. Administration of ARC183 was reported to result in the desired rapid onset of action, dose-related anticoagulation activity and rapid reversal after ceasing drug infusion. However, the amount of drug needed to achieve the desired anticoagulation activity resulted in a suboptimal dosing profile. Therefore the companies decided to pursue actively an optimized second-generation molecule. Meanwhile other groups have also developed anticoagulant aptamers such as anti-factor VIIa RNA aptamers [163] with remarkable stability ($t_{1/2}$: 15 h in plasma) and anti-factor IXa aptamers [164]. Nucleic-acid-based drugs are not as bad as their reputation. PEGylation [165] has been shown to prevent rapid elimination from the body by renal clearance [166], systemic administration is feasible, at least in animal models [167, 168] and several clinical studies have shown that aptamers are nontoxic and nonimmunogenic [165, 169]. In addition, antidotes are easy to design on the basis of Watson-Crick base-pairing rules and have proven effective in the case of the anti-factor IXa aptamer [166]. Furthermore, with the approval of the first aptamer drug (Macugen, Eyetech/Pfitzer) the way now appears to be paved for more nucleic-acid-based drugs, and so why not anticoagulative aptamers?

Other direct thrombin inhibitors reported to be in clinical trials include GW 473178 (phase II; GlaxoSmithKline) and MCC-977 (phase II; Mitsubishi Pharma). However, no structural information has been disclosed for these candidates.

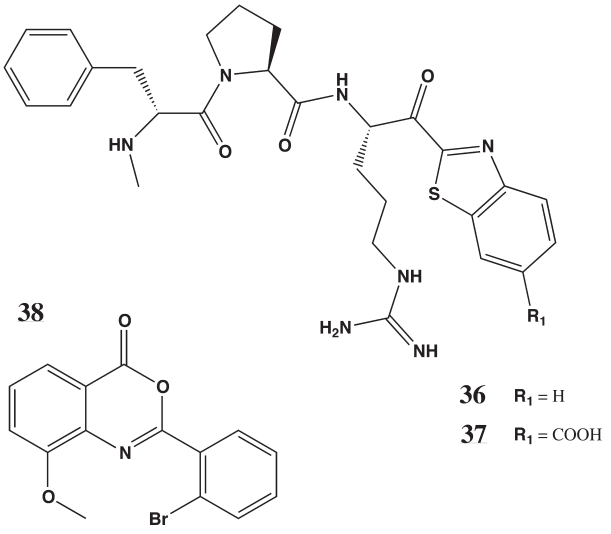

Figure 11. New direct thrombin inhibitors. Johnson & Johnson's recent direct thrombin inhibitors (**36** and **37**) and benzoxazinone-derivative HPW-RX2 (**38**).

New inhibitors – old principles

Concerning preclinical development, several groups recently reported on new direct thrombin inhibitors that are reminiscent of previous developments but nevertheless could represent promising milestones toward future drug candidates.

A group at Johnson & Johnson resumed earlier developments [170, 171] of a thrombin inhibitor that probes the S_1' binding site [172]. The design of these inhibitors is based on the idea of substituting the X in the known thrombin recognition motif D-Phe-Pro-Arg-X (see also PPACK, **9**) with heterocycles that would (i) increase the electrophilicity of the arginine carbonyl such that it would form a hemiketal adduct with the γ-oxygen of the active-site Ser195 and (ii) extend interactions with thrombin into the S_1' binding site. This work was inspired by the binding mode of cyclotheonamide (**24**, see above). Previously discovered inhibitor **36** (Fig. 11; [173]) exhibited a good antithrombin potency (K_i = 0.2 nM) with a good selectivity over key coagulation enzymes. However, selectivity over trypsin was only modest (K_i = 3.1 nM for trypsin). Furthermore, inhibitor **36** proved to be efficient in the canine arteriovenous shunt and the rabbit deep-vein thrombosis model. Unfortunately, **36** and some of its derivatives elicited pronounced hypotension and electrocardiogram effects. Further development produced inhibitor **37** which still showed good thrombin inhibitory activity (K_i = 2.0 nM). Inhibitor **37** also proved to be a potent trypsin inhibitor but was slightly less selective over coagulation enzymes compared with **36**. However, inhibitor **37** exibited significantly diminished cardiovascular side effects and had an improved profile with respect to therapeutic index [172].

The inhibitory activity of 2-substituted benzoxazinones toward serine proteases has been known for more than two decades [174–176], and structural information on inhibitor-protease complexes is available [177]. Recently, two papers described the thrombin inhibitory activity of a new synthetic benzoxazinone-derivative, HPW-RX2 **38** [178, 179]. Although HPW-RX2 **38** (Fig. 11) exhibited only moderate thrombin inhibitory activity in a standard amidolytic assay ($K_i \approx 3$ μM), it has a potent inhibitory effect on rabbit [178] and human platelet aggregation [179] induced by thrombin and arachidonic acid. Interestingly, there is experimental evidence indicating that HPW-RX2 has a dual effect inhibiting not only thrombin but also cyclooxygenase [179].

Toyota et al. [180] revived the concept of metal chelates as protease inhibitors [181] previously realized by Axys constructing thrombin inhibitors such as **39** (Fig. 12) [182, 183]. The latter was identified in a series of amidino-benzimidazoles and shows thrombin inhibition which is dependent on the presence of physiological Zn^{2+} concentrations (K_i = 5.6 nM in the presence of Zn^{2+}; K_i = 2.3 μM in the absence of free Zn^{2+}). Crystallographic analysis of the in-

hibitor-thrombin complex revealed that Zn^{2+} is bound by the side chains of thrombin residues His57 and Ser195 as well as the benzimidazole groups of inhibitor **39**. The metal ion thus acts not only as a stabilizer of the inhibitor structure but actively participates as a cofactor in inhibition. A different type of chelate was realized by Toyota et al. [180] by synthesizing Schiff bases from formyl-hydroxybenzamidines and various amino acids. Depicted are structural models of two of the most active inhibitors, **40** and **41** (Fig. 12) with inhibition equilibrium constant (K_i) of 27 nM and 410 nM, respectively. Unfortunately no further data, e.g. on selectivity, were published.

For a long time, it was widely accepted that the P1 moiety of thrombin inhibitors has to be basic in order to interact with the acidic S1 pocket, and particularly with Asp189. However, the strong basicity of many P1 groups, such as the guanidino group of arginine or the amidino group of the benzamidine residue, were assumed to cause undesired pharmacokinetic properties and unwanted side effects [184]. Although fewer basic P1 moieties do not guarantee oral bioavailability [185, 186], researchers tried to find surrogates for arginine with a less basic character. Indeed, several potent inhibitors have been identified that include P1 groups such as aminoalkyl, aminocyclohexyl [137, 187, 188], aminopyridine [32, 138, 153, 185, 189], aminopyridazine [185], aminopyrimidine [185], aminopyrazine [185], benzamidrazone [190, 191], thienylamidine [133], azaphenylalanine [192], 4,5,6,7-tetrahydro-1,3-benzothiazol-2-amine, 4,5,6,7-tetrahydro-2H-indazole and 2-imino-4,5,6,7-tetrahydro-1,3-benzothiazol-3(2H)-yl-amine [193]. Furthermore, there are also a few examples of neutral P1 moieties such as benzothiophene and hydroxybenzothiophene [194–196], cyclohexylmethyl, benzyl and dichlorobenzyl [197], 3-chlorobenzyl and 2-oxyacetamide-5-chlorobenzyl [38, 198], phenol [33], tryptophan [199] and 6-fluorotryptamine [200]. As one of the most recent examples, screening of a fragment-based compound library also resulted in the discovery of thrombin-binding fragments with a nonbasic P1 motif [201]. However, only some chlorobenzyl-based inhibitors and a few hydroxybenzothiophene derivatives such as inhibitor **42** (K_i = 0.3 nM; Fig. 13) [194–196] exhibited low-nanomolar inhibition constants. Recently, a new series of thrombin inhibitors containing D-tyrosine at the P1 position, was identified by a novel computer-assisted multiparameter optimization approach [37] based on a new type of algorithm developed by M. Thürk (Matrix Advanced Solutions Germany GmbH). The most potent candidate of this series, Goe 8–5 **43** (Fig. 13), showed good thrombin inhibitory activity (K_i = 3.0 nM) and a so far unmatched selectivity over trypsin and various coagulation enzymes (K_i > 1000 µM). Furthermore, Goe 8-5 has a favorable profile comprising nontoxicity, metabolic stability, low serum protein binding, good solubility, high anticoagulant activity and a slow and exclusively renal elimination from the circulation in a rat model. X-ray crystallographic analysis of the thrombin-inhibitor complex revealed that the OH group of D-tyrosine adapts a position nearly identical to one of the terminal nitrogens of either L-arginine in PPACK or benzamidine

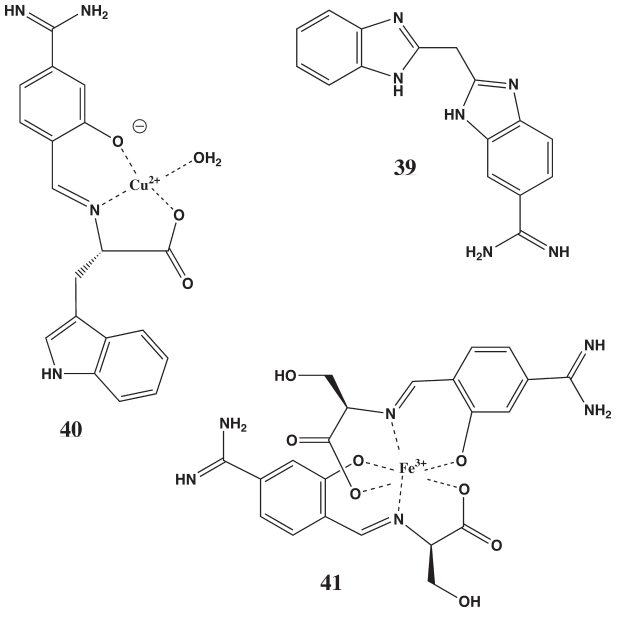

Figure 12. Metal chelates as direct thrombin inhibitors. Axys' thrombin inhibitor (**39**) and representatives from a series of Schiff bases based on formyl-hydroxybenzamidines (**40** and **41**).

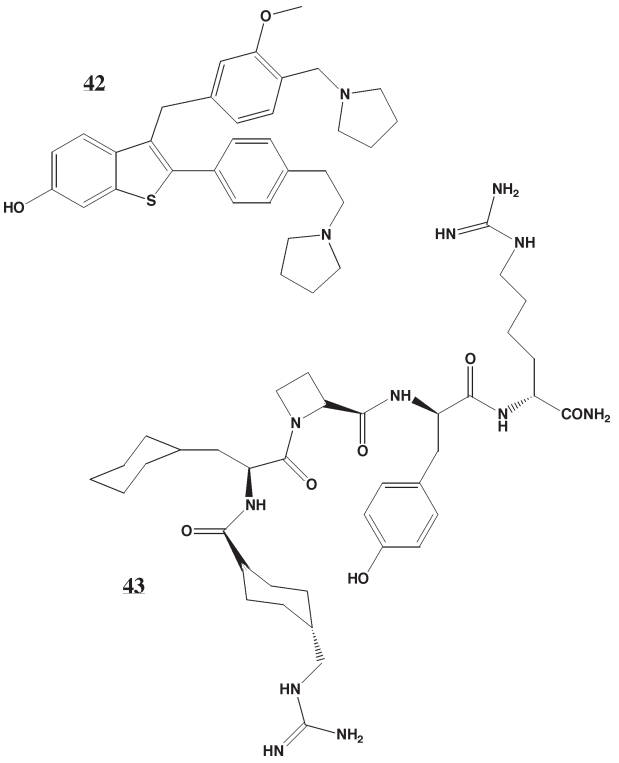

Figure 13. Direct thrombin inhibitors with a non-charged P1 moiety.

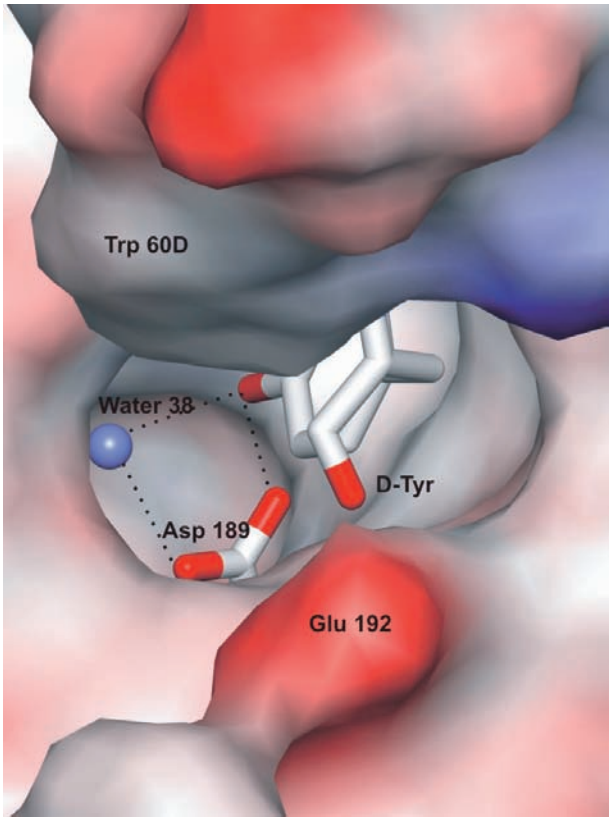

Figure 14. Mimickry of the guanidine moiety of arginine by the P1 moiety D-tyrosine and a water molecule in the structure of the Goe 8-5/thrombin complex. The OH group of D-tyrosine adopts a position nearly identical to one of the terminal side chain nitrogens of L-arginine. The other terminal nitrogen of L-arginine is substituted by a water molecule. Both, the D-Tyr-OH group and the water molecule make hydrogen bonds to the side chain oxygens of Asp189 in the S1 pocket of thrombin.

in melagatran as P1 residue. It forms a bonding network including a direct hydrogen bond to Asp189 as well as to a water molecule which connects the D-Tyr-OH group with the second carboxyl oxygen of Asp189 thus mimicking the bidentate binding mode of a guanidine moiety (Fig. 14). However, due to the kink in the D-tyrosine side chain, the side chain slots into the S1 pocket differently than an extended L-arginine or benzamidine. Therefore substitutions of D-tyrosine by L-arginine or benzamidine result in a substantial loss in antithrombotic activity [37]. Since derivatives with substitutions at the positions of basic side chains (i) show only moderate losses in thrombin inhibitory activity and (ii) these side chains do not make direct contacts to the enzyme, Goe 8–5 appears to be a promising lead for further development.

Perspectives

The development of anticoagulants is considered one of the most challenging interventions in medicine. Reasons for failure of drug candidates are manifold and include poor selectivity, inherent toxicity, high plasma protein binding, poor metabolic stability, rapid elimination from the blood, low anticoagulant activity, poor oral bioavailability and a narrow therapeutic index with the risk of bleeding complications. The case of ximelagatran shows how difficult the development of an anticoagulant drug, e.g. a direct thrombin inhibitor is, particularly in light of our great demands for safety. As a consequence, serious side effects observed in a small fraction of patients prevented final approval of a drug that really could be beneficial for the majority. Maybe it is time to reassess our principle of 'one-drug-for-everybody' and better define patients prone to developing serious complications, e.g. liver abnormalities as in the case of ximelagatran, by making more rigorous use of genomic analysis.

Despite the withdrawal of ximelagatran, a variety of novel anticoagulants with improved pharmacologic profiles are in development and there is a good chance that one of these candidates will eventually receive approval, and not only for niche indications, in the near future. However, progress has not only been made in the development of direct thrombin inhibitors. A widespread strategy is to block coagulation without affecting residual thrombin functions thus minimizing bleeding side effects. Factor Xa inhibitors have been developed as promising drug candidates, with many candidates currently in clinical trials [148, 202]. Factor Xa has been identified as an attractive target since it is upstream of thrombin in the blood coagulation cascade. As a consequence, inhibition of factor Xa efficiently prevents the generation of thrombin molecules. On average, inhibition of a single factor Xa molecule prevents the activation of more than 100 molecules of prothrombin [203]. Dual thrombin/factor Xa inhibitors [151] may be particularly efficient at this point.

Alternative strategies that use the signal amplification within the coagulation cascade aim at inhibiting coagulation even upstream of factor Xa. Drugs that target the factor VIIa/TF complex include tifacogin (recombinant TFPI) and NAPc2; drugs that block factors Va and VIIIa include activated protein C and thrombomodulin [204]. Enhancement of endogenous fibrinolytic activity could be another interesting strategy. In particular, XIIIa inhibitors as developed e.g. by Curacyte, are worth mentioning. Factor XIIIa catalyzes the covalent cross-linking of fibrin polymers and incorporation of proteins into the fibrin network and thus stabilizes the thrombus and confers relative resistance to plasmin-mediated degradation [205]. Animal experiments have demonstrated improved thrombolysis by a plasminogen activator in combination with an FXIIIa inhibitor [206]. Yet another course has been adopted by researchers at Eisei (Japan). They developed E-5555, the first oral PAR-1 antagonist, which is currently in phase I clinical trials. It is believed

that PAR-1 antagonists should show less bleeding side effects since they do not inhibit the enzymatic activity of thrombin.

In conclusion, with a significant number of new anticoagulants in late stages of development, therapeutic options for the treatment of venous and arterial thrombosis are soon likely to expand. Early ADME/Tox and pharmacokinetic testing increased the chance that these compounds can advance to late-stage clinical trials and regulatory approval, eventually replacing indirect coagulants on account of their efficacy and safety.

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