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REVIEW ARTICLE

Thrombin Active Site Inhibitors

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Abstract—Development of small molecule thrombin active site inhibitors has been an area of intense research. A brief review on recent progress and challenges is outlined.

I. Introduction

The blood coagulation enzyme thrombin plays a central role in hemostasis and thrombosis.[1] During blood coagulation, a controlled sequence of cleavages of inactive proenzymes to the active proteases occurs via the so-called intrinsic or extrinsic pathways. These enzyme cascades converge in the generation of factor Xa, which, as part of the prothrombinase complex, activates thrombin from its zymogen, prothrombin.[2] Upon activation, thrombin proteolytically cleaves fibrinopeptides A and B from the soluble plasma protein fibrinogen to give the insoluble protein fibrin. The linear fibrin monomers are then cross-linked by factor XIIIa, which is itself activated by thrombin.[3] This network of insoluble, cross-linked fibrin polymer adds mechanical stability and resistance to lysis to the resulting blood clot. Both free and clot-bound thrombin can convert fibrinogen to fibrin, thus allowing stabilization and propagation of the thrombus at a site of injury. Thrombin amplifies its own production through a positive feed back loop via proteolytic activation of two cofactors in the blood coagulation cascade, factors V and VIII. In combination with the endothelial cell surface protein, thrombomodulin, thrombin activates the serine protease protein C. Activated protein C complexes with plasma cofactor protein S and catalyzes the inactivation of factors Va and VIIIa, thus downregulating thrombin production.[4] Thrombin inactivation in plasma is achieved in part by antithrombin III, heparin cofactor II and protease nexin-1.[5,6]

Thrombin also stimulates endothelial cells to synthesize or release various antithrombotic agents such as prostacyclin,[7] tissue plasminogen activator (t-PA) and plasminogen activator inhibitor type 1 (PAI-1),[8] and endothelial derived relaxing factor (EDRF).[9] Thrombin thus plays a pivotal role in maintaining the intricate balance between hemostasis and thrombolysis. Platelet activation by thrombin leads to shape change, platelet aggregation and release of secretory granules. Thrombin activates platelets by binding to a specific receptor on the platelet surface, and cleaves between Arg41 and Ser42 in the extracellular N-terminal domain, creating a new N-terminus that serves as a tethered ligand which activates the receptor.[10] Thrombin also activates a variety of other cell types via proteolytic activation and nonenzymatic receptor-mediated processes that function with active-site blocked thrombin species.[1a,11] Thrombin stimulates adhesion of neutrophils[12] and monocytes[13] to vascular endothelium, enhances fibroblast growth factor-induced endothelial cell proliferation,[14] causes mitogenesis in macrophages, fibroblasts, leukocytes and epithelial cells,[15] and may affect growth and differentiation of cultured cells from the nervous system.[16]

In addition to its pivotal role in hemostasis and thrombosis, thrombin may be involved in atherosclerosis, inflammation and neurodegenerative diseases.[17] The discovery and development of an agent that controls the action and/or generation of this enzyme has therefore attracted considerable attention.[18]

II. Indirect Thrombin Inhibitors

Current anticoagulant therapy is limited to the indirect thrombin inhibitors—coumarins, heparins and low molecular weight heparin. Of these, only the coumarin warfarin is an effective oral anticoagulant. Warfarin is a vitamin-K antagonist that inhibits hepatic synthesis of vitamin-K dependent cofactors including factors II, VII, IX, X, thrombin and the natural anticoagulant proteins C and S.[19] This class of compounds has a long duration of action but their slow onset of action requires careful monitoring of anticoagulation in patients. In addition, warfarin has been associated with major side effects including thrombocytopenia and severe bleeding complications.

Heparin and low molecular weight heparin inhibit thrombin and other coagulation factors such as IXa, Xa, XIa and XIIa by catalyzing the inactivation of these proteases by the endogenous inhibitors antithrombin III and heparin cofactor II. Unlike warfarin, heparin has a rapid onset of action, but must be administered parenterally. Like warfarin, it suffers from haemorrhagic side effects, and because of its mechanism of action, is ineffective in patients having antithrombin III deficiency.[20] Furthermore, heparin is minimally effective in animal models of arterial thrombosis characterized by platelet dependence and high shear, possibly because of its inability to inactivate clot- and matrix-bound thrombin.[21] Low molecular weight heparins may offer some advantages over heparin with respect to side effects, but like heparin, they must be administered parenterally or subcutaneously.[22] However, clinical experience with indirect thrombin inhibitors provides the basis for the belief that a direct thrombin inhibitor may offer a safe and efficacious treatment for venous and arterial thrombosis, pulmonary embolism and restenosis following angioplasty.

III. Direct Thrombin Inhibitors

Hirudin, an acidic 65-aminoacid polypeptide isolated from the salivary gland of medicinal leech, is the most potent and selective thrombin inhibitor known. Since its characterization by Markwardt[23] in 1957, several natural variants have been isolated from the species *Hirudo medicinalis*. The tight binding of hirudin to thrombin (K_i = 0.3 pM) is achieved through interactions at both the hydrophobic binding site adjacent to the active site, and to the remote 'anion-binding exosite'.[24] Hirudin is highly selective in its binding to thrombin and has no inhibitory activity against other serine proteases. Recent advances in biotechnology have led to the cloning, microbial expression, and characterization of recombinant hirudins (r-hirudin) from *Escherichia coli*, yeast, *Saccharomyces cerevisiae*, *Bacillus subtilis* and other expression systems.[25] r-Hirudin differs from natural hirudin in lacking a sulfate group on Tyr63, which has a minor effect on potency. The superior therapeutic profile of the direct-acting hirudin compared with the indirect-acting heparin in animal models of thrombosis have made it an attractive target for drug development.[26] Recombinant hirudin is currently undergoing clinical evaluation in the GUSTO-2,[27b] TIMI-9[27a] and HIT-III[27c] trials for the treatment of acute myocardial infarction and unstable angina. Hirudin suffers from a short duration of action and more importantly, because of its peptidic nature and size, agents of this type must be administered parenterally.

The concept of an inhibitor spanning both the active site and anion-binding exosite led scientists at Biogen to develop a novel series of so-called 'hirulogs'.[28] These compounds incorporate the D-Phe-Pro-Arg sequence of active site-directed inhibitors with the anion-binding exosite residues of hirudin (Hir 53-64) linked through a spacer sequence. Hirulog-1 (D-Phe-Pro-Arg-$[Gly]_4$-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu) is a potent inhibitor of thrombin (K_i = 2.3 nM). Hirulog-1 is currently in clinical evaluation for treatment of venous thrombosis after major hip and knee surgery, deep venous thrombosis (DVT) and for prevention of acute complications of coronary angioplasty in patients with stable or unstable angina. Like hirudin, hirulog-1 is a high molecular weight polypeptide that lacks oral bioavailability and must be administered intravenously or subcutaneously.

IV. Structure in Drug Design

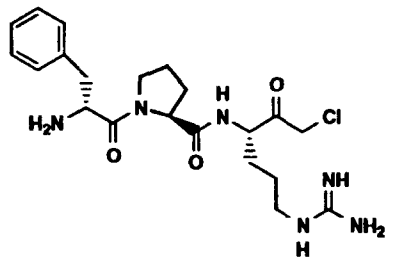
Thrombin is a member of the trypsin family of serine proteinases that include other coagulation enzymes like factors VIIa, IXa, Xa, XIa, XIIa, and protein C. This family of proteinases is characterized by a catalytic triad of Ser, His, and Asp residues at the active site. Thrombin exhibits primarily a trypsin-like specificity in that substrates with Lys or preferably Arg in the S1 site are cleaved.[29] In addition, like other members of the trypsin family, thrombin has an Asp residue in the primary substrate binding site that plays a central role in recognition and binding of substrates and inhibitors. Drug discovery research on direct-acting small molecule inhibitors of thrombin is greatly aided by the plethora of structural data that has become available. During the last few years, solid-state structures of inhibitors including D-Phe-Pro-Arg chloromethyl ketone (**1**), MD-805 (**21**), hirudin, hirulogs, and cyclotheonamide A (**16**) complexed with human α-thrombin and/or the related serine protease trypsin have been solved at high resolution and published.[30] These studies have elucidated various binding modes between different structural classes, and in some cases within classes of inhibitors. The vast wealth of information obtained from such solid-state structures is now being utilized in the design and search for more potent and selective thrombin inhibitors.

The search for an orally bioavailable, direct-acting, and specific small molecule thrombin inhibitor continues apace. Such a compound should offer superior efficacy and an enhanced risk/benefit profile compared with the existing indirect thrombin inhibitors, and also when compared with projected direct-acting thrombin inhibitors such as hirudin and hirulog.

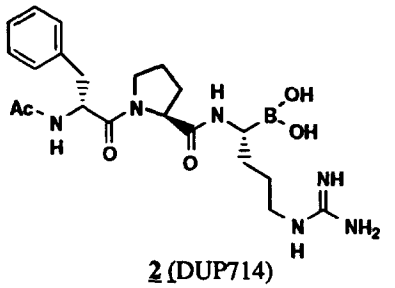
V. New Developments: Electrophilic Thrombin Inhibitors

The extreme case of an electrophilic inhibitor of thrombin is illustrated by the irreversible thrombin inactivator PPACK[31](1), which was designed based on the insight that D-Phe-Pro-Arg could mimic the sequence of thrombin's natural substrate, fibrinogen. PPACK inactivates thrombin by irreversible alkylation of the active site His57; the carbonyl group of the chloromethyl ketone forms a tetrahedral hemiketal with the active site Ser195 hydroxyl group. During the last several years a number of slow, tight binding boroArg inhibitors of thrombin have been reported, such as **2** (K_i = 41 pM),[32] and **3** (K_i = 17 pM).[33] Dissociation of the enzyme–inhibitor complex for these compounds is extremely slow with dissociation half-lives of hours to days. A major drawback of the boroArg series, lack of selectivity, has been addressed with the synthesis of nonbasic boronic esters **4** and **5**.[34] Replacement of the charged guanidino sidechain with the neutral methoxypropyl group of **4** and **5** provided compounds with a high degree selectivity for the inhibition of thrombin over plasmin, (1000-fold) and trypsin (100-fold).[17] Similarly, replacement of the guanidine moiety in **3** with a less basic amino group provided the slow-tight binding inhibitor **6** (K_i = 39 pM)[35] which has a 10-fold selectivity over trypsin and 90-fold selectivity over plasmin. The potent antithrombotic activities of **4–6** suggest that the charged interaction of the guanidine moiety with Asp189 in the specificity pocket is not an essential requirement for direct-acting thrombin inhibitors. In analogy to the boronates, phosphonic acid ester **7** (K_i = 4.8 nM) inactivates thrombin by initial nucleophilic attack of Ser195 hydroxyl group on phosphorus to form a pentavalent phosphorus intermediate which is slowly converted to a more stable tetrahedral inhibitor–thrombin complex.[36]

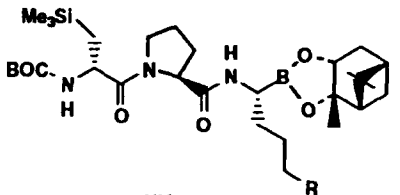
A large number of electrophilic inhibitors have been prepared which incorporate an activated carbonyl group at the site of the scissile bond. Electrophilic aldehydes, ket-



1 (PPACK)



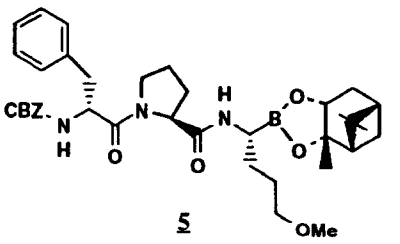
2 (DUP714)



3 R = HN=C(NH)NH$_2$ (SDZ 217-766)

4 R = OMe (SDZ 219-349)

6 R = CH_2NH_2 (SDZ MTH 958)



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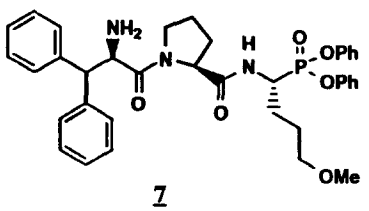

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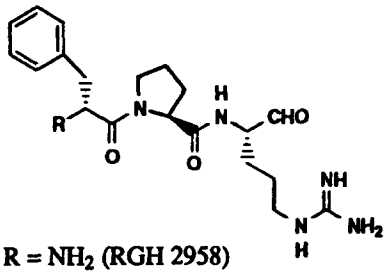
Figure 1. Electrophilic thrombin inhibitors.

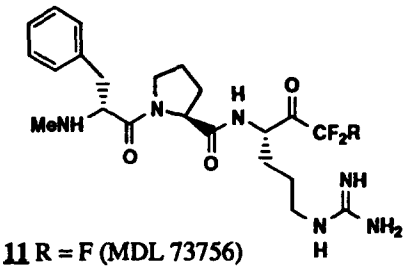
ones, activated fluoromethyl ketones, α-ketoesters, and amides form tetrahedral intermediates with the thrombin active site Ser195 hydroxyl group, thereby inhibiting the enzyme. The first examples of this class are the aldehydes D-Phe-Pro-Arg-H **8** and its more stable *N*-methyl analog **9**. Both compounds are slow, tight-binding inhibitors of thrombin.[37] Compound **9** (GYKI 14766, Efegatran) has proven to be an effective antithrombotic agent in animal models and is currently in clinical development.[38] Extensive SAR studies of the P_3 residue have been reported.[39] In contrast to **8** and **9**, the des-amino analog **10**[40] is a rapid-acting competitive inhibitor of thrombin (K_i = 140 nM) and does not exhibit time-dependent inhibition kinetics. This compound is efficacious in animal models of arterial and venous thrombosis.[41]

Based on analogy with other protease inhibitors, trifluoromethyl ketone **11** (K_i < 1 nM), and its analogs **12** (K_i = 80 nM) and **13** (K_i = 5 nM)[42] were designed as time-dependent slow, tight-binding inhibitors of thrombin. Although the trifluoromethyl ketone **11** is the most potent of these inhibitors *in vitro*, the difluorobutyl analog **13** is the most potent in a rat model of $FeCl_3$-induced arterial thrombosis. The D-Phe-Pro-Lys α-keto ester **14**, and its methanol addition adduct **15** (K_i = 1.7 nM)[43] were also prepared as slow, tight-binding inhibitors of thrombin.

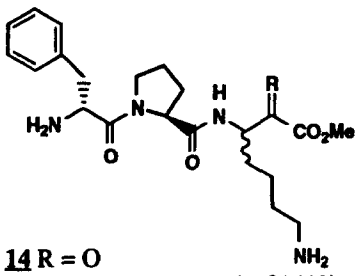
The macrocyclic peptides cyclotheonamide A (CtA, **16**) and cyclotheonamide B (CtB, **17**) are electrophilic thrombin inhibitors with an unusual composition isolated from the marine sponge *Theonella* sp.[44] The total synth-



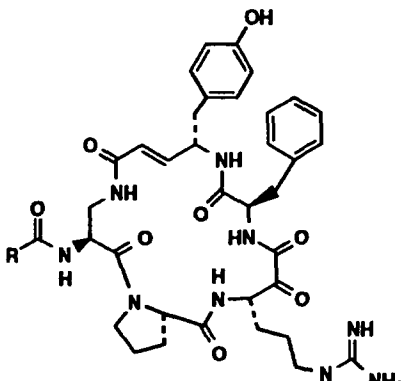
8 R = NH_2 (RGH 2958)
9 R = NHMe (GYKI 14766)
10 R = H (BMY 44621)



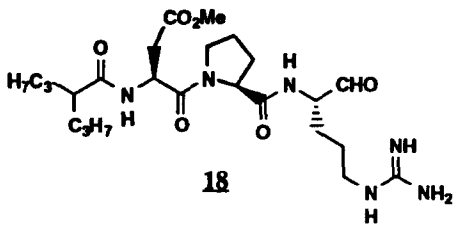
11 R = F (MDL 73756)
12 R = CF_3 (MDL 74063)
13 R = n-C_3H_7 (MDL 75579)



14 R = O
15 R =OH, OMe (BMS 181412)



16 R = H (Cyclotheonamide A)
17 R =Me (Cyclotheonamide B)



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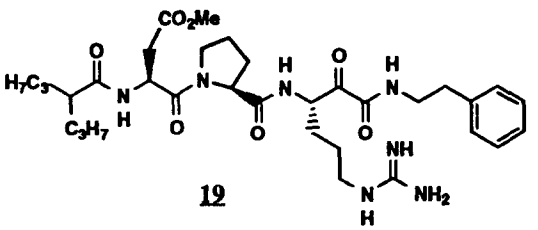

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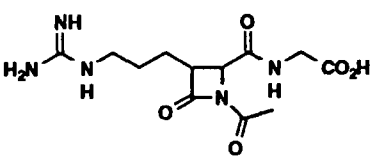

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Figure 2. Electrophilic thrombin inhibitors.

eses of both CtA and CtB, and X-ray crystal structures of CtA bound to thrombin[45] and trypsin[46] have been reported. The CtA–thrombin/trypsin complexes display a 'typical' Pro-Arg interaction at the S_2 and S_1 sites. In addition, the ketone group of the keto-amide interacts with Ser195 hydroxyl group to form a tetrahedral hemiketal intermediate that resembles the intermediate for peptide hydrolysis. These are the first solid state structures of an α-keto-amide complex of a serine protease. CtA is not selective for the inhibition of thrombin (K_i = 180 nM) compared with plasmin (K_i = 370 nM), trypsin (K_i = 23 nM), or streptokinase (K_i = 35 nM).

A variation on the tripeptide aldehyde theme incorporates an L-Asp residue in place of D-Phe at P_3 (**18**, K_i = 1.2 nM); the corresponding keto-amide **19** (K_i = 0.48 nM) is also reported as a potent thrombin inhibitor.[47] A novel series of β-lactam inhibitors of thrombin such as **20** have also been reported.[48]

VI. New Developments: Non-electrophilic Thrombin Inhibitors

As the title suggests, this collection of inhibitors lacks an electrophilic group capable of forming a covalent tetrahedral intermediate with active site Ser195 of thrombin. Unlike most of the electrophilic inhibitors, these compounds exhibit classical competitive kinetics with no time dependence of inhibition. The first prototypes of this class, MD-805 (**21**, K_i = 19 nM) and NAPAP (**22**, K_i = 6 nM) are the result of extensive SAR studies with piperidine amide derivatives of N^{α}-dansyl-L-arginine,[18h] and N^{α}-arylsulfonyl benzamidines,[49] respectively. MD-805 is one of the most selective thrombin inhibitors known, and is marketed in Japan as an intravenous agent. It is in development in Europe and the US for the treatment of myocardial infarction, angioplasty and unstable angina. Recent studies have demonstrated the superiority of MD-805 over heparin and r-hirudin in inhibiting fibrin- and clot-bound thrombin; this finding may have important implications in thrombolytic therapy for acute MI.[50] X-Ray crystal studies of MD-805 and NAPAP (**22**) bound to human α-thrombin[30] have shown that these compounds bind to thrombin in a more compact U-type conformation and the arginyl/*p*-amidinophenylalanine residues enter the specificity pocket from a different direction than **1**. This structural information was utilized recently in designing the potent and selective agent **23**, (K_i = 270 pM) which is 7000-fold selective for thrombin over trypsin.[51] The preparation of direct analogs of MD-805 has also been an active area of research; several analogs replacing the basic guanidino moiety of MD-805 with imidazole, thioimidazole, benzimidazole, and amino-pyridine have been reported in the patent literature.[52] The glucuronylamino-analog **24** related to NAPAP is claimed to be a metabolically stable, potent and selective thrombin inhibitor (K_i = 0.085 nM).[53]

A novel class of non-electrophilic thrombin active site inhibitors was obtained by optimization of an in-house screening lead at Bristol-Myers Squibb. The most potent compound reported in the series, **25** (K_i = 22 nM) displays better selectivity for thrombin versus trypsin and plasmin than **9**.[54] SAR studies combined with modeled bound conformation of this new series suggest that the peptide backbone binds in a reverse fashion compared to natural substrate or inhibitors designed on the D-Phe-Pro-Arg sequence. In this 'retro' binding mode, the amide backbone forms parallel β-hydrogen bonds with Gly216 positioning the 4-guanidinobutanoyl side chain in the primary specificity pocket and the amino acid side chains oriented in a fashion similar to the first three amino acid residues of hirudin.

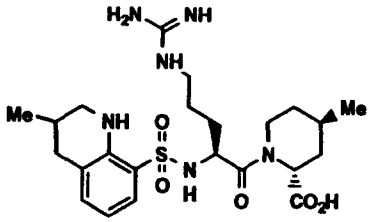
Workers at Astra[55] have reported tripeptide thrombin inhibitors related to D-Phe-Pro-Agm (**26**), which lack the electrophilic aldehyde group of compound **8**. Astra's compound **27** appends an N-terminal acetic acid to the tripeptide motif.

VII. Challenges

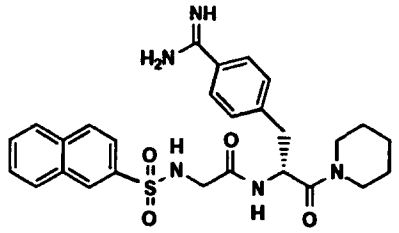
The direct-acting thrombin inhibitors hirudin and hirulog are clinically effective in acute care settings. However, the larger market for the prevention of myocardial infarct and stroke requires long-term oral dosing of an anticoagulant. The indirect-acting anticoagulant warfarin is effective in the secondary prevention of myocardial infarction, venous thromboembolism, and stroke;[19] it will only be displaced by improving upon the narrow therapeutic index and patient monitoring requirements of warfarin.

The challenge that the development of direct thrombin inhibitors poses for the medicinal chemist may be taken from a recent discussion by Sixma and de Groot:[56] "An ideal antithrombotic drug should inhibit thrombosis without affecting hemostasis. It should have a long half life. It should be absorbed after oral administration; it should be safe and it should have a wide therapeutic range". These are the hurdles that must be overcome in the course of successfully discovering and developing a clinically useful small molecule thrombin inhibitor.

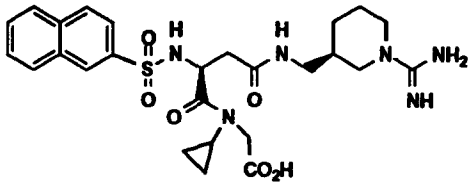
Safety is a key issue for chronic use outside the hospital setting. High oral bioavailability and a long half life will be important in allowing predictable peak/trough ratios of a thrombin inhibitor to be maintained. Once or twice-daily dosing of a long acting compound may minimize the need for monitoring the level of anticoagulation. To our knowledge, none of the thrombin active site inhibitors described in this review possess adequate half-lives for an oral dosing regimen. Selective inhibition of thrombin with respect to other serine proteases is also essential if a safe and effective drug is to be developed. It has recently been demonstrated in an animal model of venous thrombosis that electrophilic thrombin inhibitors **2** and **9** are capable of inhibiting streptokinase-induced clot lysis.[57] The resulting 'fibrinolytic compromise' demonstrates a potential danger—that an inadequately selective thrombin active site inhibitor might under some conditions actually promote hemostasis. Currently, it appears that non-electrophilic compounds related to **21** and **22** are signifi-



21 (MD-805)



22 (NAPAP)



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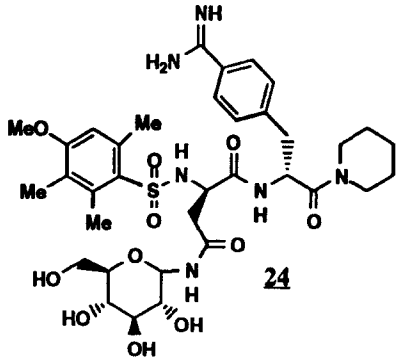

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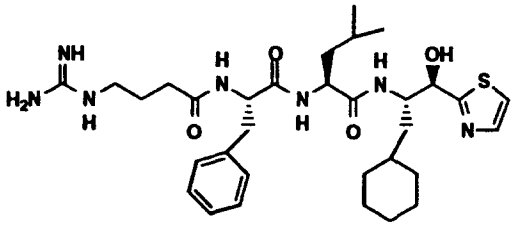

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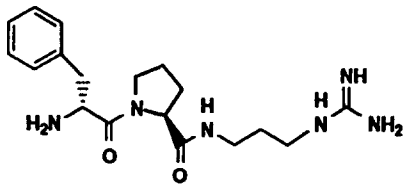

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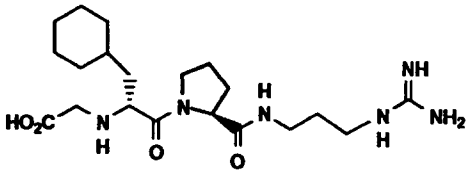

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Figure 3. Non-electrophilic thrombin inhibitors.

cantly more selective than electrophilic D-Phe-Pro-Arg species.

An effective thrombin inhibitor must further have appropriate pharmacodynamics. The pharmacodynamic properties of boronic acid thrombin inhibitors have been discussed by Tapperelli *et al.*[34a] who have argued that the rate of association of the inhibitor with thrombin may be critical to its anticoagulant and antiplatelet activity *in vivo*. The reversible (non-electrophilic) inhibitors argatroban **21** and NAPAP **22** more rapidly and completely inhibited platelet accumulation in the rat than the boronic ester **5**. This result indicates a possible shortcoming that may be particular to electrophilic thrombin inhibitors although further research along these lines is necessary. Issues of bioavailability, half life, selectivity, and pharmacodynamics can only be resolved by the continued vigorous design, discovery, and pursuit of small molecule direct-acting thrombin inhibitors.

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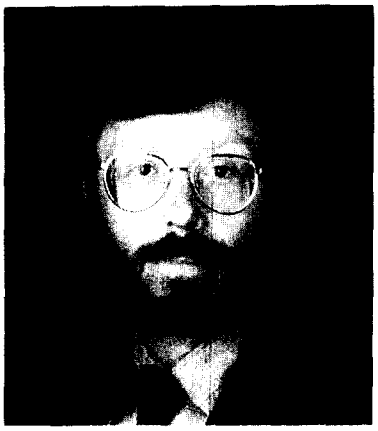

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