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THROMBIN INHIBITORS AS ANTITHROMBOTIC AGENTS: THE IMPORTANCE OF RAPID INHIBITION

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For use as an antithrombotic agent, a thrombin inhibitor must be potent and specific, i.e., it should not significantly inhibit the proteases of the anticoagulation (activated protein C) and fibrinolytic systems (plasminogen activator and plasmin). Previous evaluation of potency and specificity has been based on inhibition constants (K_i values). However, consideration of the kinetic parameters for natural plasma serine protease inhibitors indicates that a low K_i value with thrombin is not sufficient; the inhibited complex must also form rapidly. Moreover, potent inhibition of activated protein C and plasmin could be tolerated providing the inhibited complex only forms slowly. An ideal profile of kinetic parameters with thrombin, activated protein C and plasmin is formulated and discussed in relation to various classes of thrombin inhibitors. Examination of kinetic data for thrombin inhibitors currently in clinical trials (hirudin and hirulog) indicates that they possess this ideal profile of kinetic parameters.

KEY WORDS: Thrombin, inhibitor, kinetics, antithrombotic agent

THE BLOOD COAGULATION SYSTEM AND ITS CONTROL

Thrombin plays a pivotal role in both the platelet activation and fibrin generation inherent to thrombosis. In the blood, thrombin is produced through the action of the prothrombinase complex (Factors Xa, Va, Ca^{2+} and phospholipid). Once generated, thrombin cleaves fibrinogen to give fibrin monomers which polymerize to form the matrix of the clot. Thrombin further stimulates its own production by activating factors V, VIII and platelets.[1] It also activates factor XIII which then stabilizes the clot by cross-linking the fibrin. In addition, thrombin-induced platelet aggregation contributes to the body of the clot and provides a surface for the assembly of the protease-cofactor complexes of the coagulation cascade.

The procoagulant activities of thrombin are blocked by the binding of thrombin to the endothelial cell surface protein thrombomodulin. In complex with thrombomodulin, thrombin becomes a more efficient activator of protein C, a protease with anticoagulant activity. In addition, the thrombomodulin-thrombin complex is

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no longer able to cleave fibrinogen and factor V, and cannot activate platelets. Furthermore, activated protein C (APC) "turns off" the coagulation cascade by inactivating factors Va and VIIIa which are essential cofactors for the formation of thrombin.[2] Clot formation is also limited by the fibrinolytic system. Plasminogen is converted to plasmin by tissue-plasminogen activator and plasmin degrades the fibrin network of the clot leading to its dissolution.

Thus, haemostasis depends on a balance between the activity of thrombin, which promotes clot formation, and of APC and plasmin, which prevent excessive clotting. This balance needs to be kept in mind when designing thrombin inhibitors; they should control thrombin's activity without interfering with the activities of APC and plasmin.

STRUCTURAL BASIS FOR THROMBIN'S SPECIFICITY

Since the structural basis for thrombin's specificity has been reviewed in detail elsewhere,[3,4] the present article will only examine this topic briefly. Residues of substrates of proteases are usually numbered from the cleaved bond as follows: P_n-...-P_3-P_2-P_1-P'_1-P'_2-P'_3-···-P'_n where cleavage occurs at the P_1-P'_1 bond; the corresponding binding sites for these residues on the protease are designated as S or S' sites. Although thrombin displays considerable structural homology to trypsin and like this protease prefers basic P_1 residues, its specificity is much more restricted. This restricted specificity of thrombin has been achieved by modifications of the active-site structure and by using other regions (called exosites) in its interactions with substrates, cofactors and inhibitors.

The binding sites for residues on the N-terminal side of the scissile bond were defined by the crystal structure of thrombin with D-Phe-Pro-ArgCH_2Cl (PPACK), a potent inhibitor that inactivates thrombin by alkylating the active-site histidine[5] (Figure 1). The P_1 arginine of PPACK binds to the primary specificity pocket or S_1 site which consists of a deep channel with Asp189 located at the bottom. Asp189 forms a salt bridge with the P_1 arginine which establishes thrombin's preference for basic residues in the P_1 position. In comparison with trypsin, the S_1 site is more hydrophobic and this has allowed the development of thrombin inhibitors with uncharged P_1 residues which show good selectivity with respect to other trypsin-like serine proteases.[6] A large insertion in a loop on the edge of the active site of thrombin restricts access to the active site and creates a particular hydrophobic S_2 binding site (Figure 1). This S_2 site, formed in part by Tyr60A and Trp60D from the insertion loop, is well adapted for the binding of proline and most synthetic peptide inhibitors of thrombin have incorporated a proline residue in the P_2 position.[7] The P_3 D-phenylalanine of PPACK binds to the S_3 site constructed by Leu99, Ile174 and Trp215. Interactions with Trp215 make this site particularly suitable for aromatic residues and it has been termed the "aryl-binding" site. It is also occupied by aromatic residues in the complexes of thrombin with other ligands including hirudin, fibrinopeptide A and synthetic inhibitors[4] (Figure 1). The aryl-binding site is smaller and more hydrophobic than the corresponding site in trypsin and it should be possible to use interactions with this site in the design of specific thrombin inhibitors.

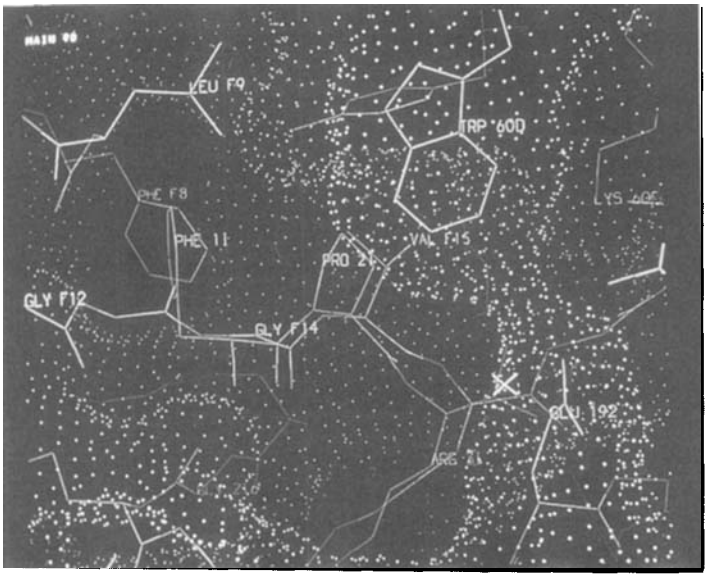

FIGURE 1 Binding of PPACK and fibrinopeptide A to the active site of thrombin. The active site of thrombin (green) is displayed along with its Connolly surface. The binding of an analog of the substrate fibrinopeptide A (yellow) and the inhibitor PPACK (crimson) are shown; PPACK and fibrinopeptide A residues are prefixed by "I" and "F", respectively. The P_1 arginine residues penetrate in the channel-like primary-specificity pocket. The P_2 proline of PPACK binds to the hydrophobic S_2 site that is formed in part by Trp60D from the insertion loop. The "aryl-binding" pocket is occupied by the P_3 D-phenylalanine of PPACK. The peptide chain of fibrinopeptide A leaves the active site after GlyF14 and returns in the opposite orientation allowing the aromatic ring of PheF8 to occupy a similar position to that of the D-phenylalanine of PPACK.

The most important exosite contributing to thrombin's specificity is a surface groove rich in basic amino acids that has been termed the anion-binding exosite or the fibrinogen-recognition exosite.[4] The anion-binding exosite has been implicated in the interaction of thrombin with fibrinogen, heparin cofactor II, the thrombin receptor and thrombomodulin as well as the inhibitor hirudin and hirudin analogues.[4] This binding site on thrombin was originally defined by structures of thrombin-hirudin complexes in which the negatively charged C-terminal region of hirudin was shown to be bound to the anion-binding exosite[8,9] (Figure 2). Electrostatic calculations indicate that the anion-binding exosite has a large positive potential centred around the thrombin loop Lys70-Glu80 with up to nine basic residues contributing to the field. This field interacts with the negative potential created by the seven negatively charged groups in the C-terminal region of hirudin.[10] Hydrophobic interaction serve to anchor the exosite-binding peptides in an appropriate orientation for alignment of the electrostatic fields (Figure 2). Phe56' of hirudin is buried in an aromatic binding pocket with the aromatic side chains of Phe56' and Phe34 making a favourable edge-to-face interaction. Other hirudin residues (Pro60', Tyr63' and Leu64') are also involved in hydrophobic interactions (Figure 2).

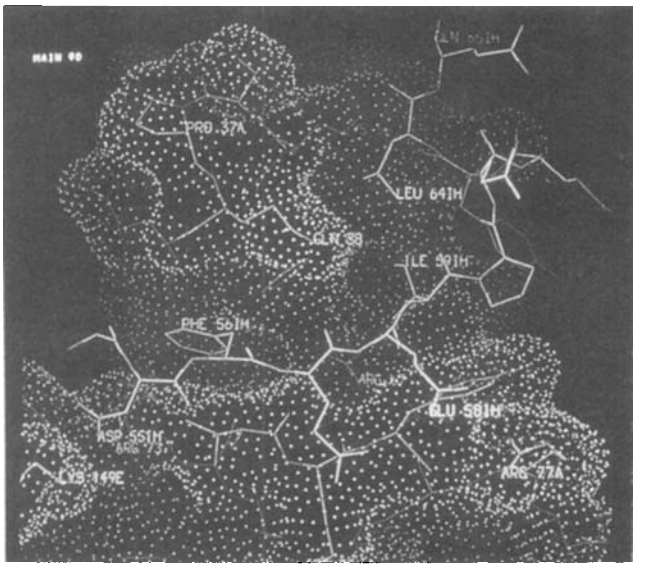

FIGURE 2 Binding of the C-terminal tail of hirudin to the anion-binding exosite. The binding of hirudin (yellow) to the anion-binding exosite of thrombin (green) is displayed along with thrombin's Connolly surface. Hirudin residues are denoted by the "IH" suffix. The hirudin tail binds in an extended conformation before ending in a 3_{10}-helix and fits snugly into the anion-binding exosite. Negatively charged hirudin residues at positions 55, 57, 58, 61 and 62 contribute to the negatively electrostatic potential of hirudin's C-terminal tail. The van der Waals contacts made by Phe56IH, Leu59IH, Pro60IH and Leu64IH are important in anchoring the hirudin tail to the exosite.

LIMITATIONS OF CURRENT ANTITHROMBOTICS

The activity of heparin, the current mainstay of antithrombotic therapy, depends on the presence of cofactors. It acts primarily by accelerating the rate at which antithrombin inactivates thrombin and factor Xa. In addition, heparin also accelerates the rate of inhibition of thrombin by heparin cofactor II. Although heparin is very effective in the prevention and treatment of thromboembolic disorders, it has certain limitations.[11] Patients have an extremely variable response to heparin which necessitates careful laboratory monitoring to ensure that an adequate anticoagulation response is achieved without undue risk of bleeding. Heparin is also ineffective in patients with antithrombin deficiency. The variable response to heparin is at least partly due to its binding to plasma proteins including fibronectin, vitronectin and von Willebrand factor. Since some of the heparin-binding proteins are acute-phase reactants, the amount of nonspecific sequestration of heparin will increase during illness.[11] Heparin can also be inactivated by platelet factor 4 and heparinase, which are released from activated platelets.

One of the most serious limitations of heparin is its inability to catalyze the inactivation of clot-bound thrombin. After converting fibrinogen to fibrin, thrombin remains bound to fibrin via a site distinct from its catalytic centre and anion-binding exosite and becomes resistant to inactivation by the heparin-antithrombin complex. Clot-bound thrombin can, however, cleave fibrinogen and activate factors V and VIII and platelets. This can lead to persistent activation of the coagulation cascade at sites of thrombus formation.[12] This resistance of clot-bound thrombin to heparin-antithrombin is also important during thrombolytic therapy, since thrombin bound to the fibrin degradation products is also protected from inhibition by antithrombin and, thus, could cause rethrombosis.[13] At sites of vascular injury, thrombin can bind to the extracellular matrix produced by endothelial cells and the results of Bar Shavit *et al.* suggest that matrix-bound thrombin is also resistant to inactivation by antithrombin.[14] Although the newly introduced low molecular weight heparins exhibit less non-specific binding to plasma proteins, a greater bio-availability and a more predictable anticoagulant response,[15] clot-bound thrombin will be still resistant to antithrombin in the presence of these heparins.[11] In contrast to heparin, direct thrombin inhibitors, such as PPACK, hirulog and hirudin can inactivate the thrombin bound to fibrin.[12]

KINETIC PARAMETERS OF AN IDEAL THROMBIN INHIBITOR

Affinity requirements

It has been calculated that thrombin concentrations as high as 140 nM can be generated upon vascular injury.[16] Immediately after the initiation of the coagulation cascade, however, the concentration of thrombin will be lower and these low-nanomolar concentrations of thrombin can cause platelet aggregation and amplify the reactions leading to clot formation. Thus, the plasma concentration of inhibitors should ideally be at least 100 nM, but lower levels may hinder thrombin-induced platelet aggregation and the concomitant amplification of the coagulation cascade. The degree of inhibition of thrombin by these concentrations of inhibitors will depend on their effective inhibition constant ($K_{i'}$). The effective inhibition constant is obtained by correcting the true constant (K_i) for the effect of competing substrate (S) by using the formula:

$$K_{i'} = K_i.(1 + [S]/K_m)$$

where K_m is the Michaelis constant for the substrate. In plasma, the main competing substrate will be fibrinogen which is present at a concentration roughly equal to its Michaelis constant[17] and, thus, the effective inhibition constants for inhibitors will be about two-fold higher than the true constants. As a rule of thumb, the $K_{i'}$ value should be 10-fold lower than the inhibitor concentration in order to obtain effective (90%) inhibition. Thus, if a plasma inhibitor concentration of 100 nM can be achieved, a $K_{i'}$ value of 10 nM should be adequate to control thrombin activity. If the plasma concentration of inhibitor is lower, tighter binding will be required to guarantee adequate inhibition.

Tight-binding is insufficient – rapid inhibition is also required

Although the effective inhibition constant governs the amount of a complex that will be formed at equilibrium, tight-binding inhibition is not sufficient to ensure an antithrombotic effect. Antithrombin is the best example of an inhibitor that binds tightly to thrombin but does not inhibit it sufficiently rapidly to prevent thrombosis under certain circumstances. Although the inhibition constant for antithrombin is less than 1 nM, its association rate constant (k_{ass}) is only about 1×10^4 $M^{-1}s^{-1}$ (ref. 18,19). The rate at which the inhibited complex forms will be equal to $k_{ass} \cdot [I]$, where [I] is the concentration of the inhibitor. The plasma concentration of antithrombin is 2.3 μM[20] and, thus, rate for the formation of the thrombin-antithrombin complex will be 0.03 s^{-1}. The half-life ($t_{1/2}$) for the formation of the thrombin-antithrombin complex is given by $\ln(2)/k_{ass} \cdot [I])$ and is equal to about 30 s. Since the rate of inhibition of thrombin by antithrombin during thrombus formation is too slow, antithrombotics should aim to achieve a shorter half-life for the inhibition of thrombin.

In the presence of heparin, antithrombin becomes a very rapid inhibitor of thrombin and despite its limitations with respect to inhibition of clot-bound thrombin (see above), heparin is a very effective antithrombotic in many circumstances. Thus, the rate of inhibition of soluble thrombin by the heparin-antithrombin complex represents a target rate for other antithrombotics. In the presence of an optimal concentration of heparin, the k_{ass} value for antithrombin is about $(1.5–4) \times 10^7$ $M^{-1}s^{-1}$ (ref. 19) and, thus, the rate of complex formation would be 30–90 s^{-1} which corresponds to a half-life of 8–24 ms. This rate of inhibition is, however, unlikely to be achieved during anticoagulation with heparin. Heparin stimulates the rate of inhibition by simultaneously binding one molecule of antithrombin and thrombin; the optimal rate is obtained when the concentration of heparin is equal to or slightly greater than that of antithrombin. The therapeutic concentration of heparin is, however, much lower; plasma levels are usually 0.3–0.6 units/ml which corresponds to a concentration of about 0.1–0.2 μM. The concentration of the inhibitory form of antithrombin will equal this concentration and, thus, the rate of complex formation will be about 3–8 s^{-1} ($t_{1/2} \approx 0.1–0.2$ s). These values can be used to calculate the target k_{ass} values for thrombin inhibitors. If the plasma concentration of inhibitor is 0.1 μM, the target k_{ass} would be 1×10^8 $M^{-1}s^{-1}$. Two of the thrombin inhibitors (hirudin and hirulog) currently in clinical trials have k_{ass} values in this range.[21,22] Values for k_{ass} are not available for many of the low molecular weight inhibitors. Several values have been determined at 25°C and were in the range of 10^7 $M^{-1}s^{-1}$ (ref. 6); these values should increase with temperature and may be close to the target k_{ass} of 10^8 $M^{-1}s^{-1}$ at 37°C.

The importance of rapid inhibition of thrombin has been confirmed using a series of low molecular weight inhibitors of thrombin. The ability of these inhibitors to prevent thrombin-induced platelet aggregation was found to be correlated with their k_{ass} values rather than with the K_i values.[6] The results of these studies suggest, however, that the above calculations based on antithrombin and heparin may overestimate the target k_{ass}; a half-life for complex formation of 1–10 s may be adequate and this would correspond to k_{ass} values of 10^6–10^7 $M^{-1}s^{-1}$ with a plasma concentration of 0.1 μM.

Selectivity requirements

Antithrombotics should not inhibit proteases of the anticoagulant and fibrinolytic systems, i.e., the compounds should display minimal inhibitory activity with APC and plasmin. A low level of inhibition will be achieved if the K_i values with these proteases are less than 10% of the plasma concentration of the inhibitor. Thus, assuming a plasma concentration of 0.1 μM, the K_i values of the inhibitors with APC and plasmin should be greater than 1 μM. A high K_i value for plasmin is particularly important if the inhibitors are to be used in thrombolytic therapy since any inhibition of plasmin will reduce the rate of clot lysis. Hirudin and a number of the low molecular weight inhibitors have suitably low affinity for plasmin. Less information is available on inhibition of APC.

Above it was argued that the rate of inhibition of thrombin was important in obtaining an antithrombotic effect. A similar argument can be applied to the interactions of the inhibitors with APC and plasmin. Once again, the example of antithrombin can be used. Although antithrombin forms tight complexes with APC and plasmin, the rate of formation of the complexes is slow and, thus, antithrombin is not a major inhibitor of either protease.[23,24] The rate of inhibition may be an important consideration for mechanism-based thrombin inhibitors such as chloromethylketones and boronates. Like serpins, these compounds form tight complexes with a range of poteases but the rate of formation of the complexes varies.

In plasma, the activities of APC and plasmin are controlled by the serpins protein C inhibitor (PCI) and α_2-antiplasmin, respectively. Antithrombotics should not augment the rate of inhibition of these two proteases. The k_{ass} value for PCI with APC is about $7 \times 10^2 M^{-1}s^{-1}$ (ref. 24) and the plasma concentration of the inhibitor is 0.1 μM[25] which yields a rate for the formation of the inhibited complex of $7 \times 10^{-5}s^{-1}$ with a corresponding half-life of 160 min. In the presence of heparin, the k_{ass} for PCI with APC is moderately increased to 1.5×10^4 $M^{-1}s^{-1}$ (ref. 24) and the rate of complex formation will be $1.5 \times 10^{-3}s^{-1}$ ($t_{1/2} \approx 10$ min). An inhibitor will not significantly increase the intrinsic rate of inhibition of APC provided it reacts more slowly than PCI. With a plasma concentration of inhibitor roughly equal to that of PCI (0.1 μM), its k_{ass} value should be less than 7×10^2 or 1.5×10^4 $M^{-1}s^{-1}$ depending on whether the inactivation rate in the presence of heparin is considered appropriate. Data obtained with another serpin (α_1-antitrypsin with a P_1 arginine or Arg-α_1-antitrypsin) in a baboon model of septic shock suggest that the k_{ass} value in the absence of heparin may be a more appropriate upper limit. Activation of coagulation is the main cause of death associated with septic shock and a protective effect for APC has been demonstrated.[26] Although Arg-α_1-antitrypsin is an effective inhibitor of thrombin, it was unable to prevent the activation of coagulation in septic shock.[27] In addition to inactivating thrombin, Arg-α_1-antitrypsin is an effective inhibitor of APC with a k_{ass} value of 7×10^4 $M^{-1}s^{-1}$ (ref. 24). Thus, administration of Arg-α_1-antitrypsin inhibited the APC anticoagulant pathway and persistent activation of the coagulation cascade occurred instead of the inhibition that would be required for amelioration of the shock.

The k_{ass} value for α_2-antiplasmin with plasmin is 2×10^7 $M^{-1}s^{-1}$ and its plasma concentration is 1 μM[28] which will yield a rate of inhibition of 20 s^{-1} ($t_{1/2}$ = 35 ms).

TABLE 1
Ideal Kinetic Parameters for a Thrombin Inhibitor to be Used as an Antithrombotic Agent

Protease	K_i (nM)		k_{ass} ($M^{-1}s^{-1}$)
Thrombin	≤ 10	and	$\geq 10^7$
APC	≥ 1000	or	$\leq 10^3$
Plasmin	≥ 1000	or	10^6

Thus, at a concentration of 0.1 μM, inhibitors with a k_{ass} value less than 2×10^6 $M^{-1}s^{-1}$ will not augment the intrinsic rate of inactivation of plasmin.

The kinetic requirements for the ideal thrombin inhibitor are summarized in Table 1.

REPRESENTATIVES FROM DIFFERENT CLASSES OF INHIBITOR HAVE THE DESIRED KINETIC CHARACTERISTICS

Many groups have been involved in the development of thrombin inhibitors as potential antithrombotic agents. In addition to the above kinetic profile, the inhibitor should display a suitable pharmacological profile: low toxicity, reasonable plasma half-life and, if possible, oral availability. Thrombin's extended binding regions allow the use of a number of different strategies to obtain suitable inhibitors and it has been possible to design inhibitors that bind to the active site, the anion-binding exosite or both.

Active-site inhibitors

There has been considerable interest over the past two decades in mechanism-based inhibitors of thrombin. These inhibitors interact with the catalytic residues of thrombin to form a tight, often irreversible, complex. A reactive group attached to the P_1 residue interacts with the catalytic serine and/or histidine and specificity is achieved by modification of the P_1-P_3 residues. The archetypal mechanism-based inhibitor is PPACK.[29] The fit of the P_1-P_3 residues of PPACK to the S_1-S_3 sites is almost optimal and the chloromethylketone irreversibly alkylates the active-site histidine[5] (Figure 1). The result is a potent, selective inhibitor of thrombin. The kinetic profile of PPACK fits well with that outlined in Table 1; it inactivates thrombin rapidly but acts more slowly with plasmin and APC.[30,31] The major disadvantage of PPACK is the chemical reactivity of the chloromethylketone moiety; this group readily alkylates free cysteines with the result that other proteins in plasma are non-specifically modified by this reagent. Thus, although PPACK has been a useful reagent for proving the advantages of a direct thrombin inhibitor *in vivo*,[32] it is not suitable for therapeutic use.

Boronic acids and esters have been investigated as alternative reactive groups. DUP714 is based on the D-Phe-Pro-Arg sequence and is the most well studied

example of this class. The kinetic parameters for inhibition of thrombin match those of the ideal inhibitor outlined in Table 1; its K_i value in the picomolar range and the k_{ass} value is greater than 10^7 $M^{-1}s^{-1}$ (ref. 33). DUP714 is an effective antithrombotic in experimental models of arterial and venous thrombosis[34] and it shows some oral bioavailability[7]. Although the kinetic parameters of DUP714 with APC and plasmin have not been determined in detail, there appear to be potential problems with selectivity. DUP714 inhibits plasmin reasonably well and this will limit its use in thrombolytic therapy where thrombin inhibitors are used together with thrombolytic agents to prevent rethrombosis once the fibrin clot has been dissolved.

The extremely tight binding of the boronic acid analogues to thrombin is due largely to the interaction of the boronate with the active site serine and, consequently, it has been possible to modify other substituents of the inhibitor in order to achieve selectivity. Interestingly, it was possible to substitute the charged arginine of the peptide boronates with a neutral side chain, such as methoxypropyl, and still retain reasonable inhibitory potency with respect to thrombin while increasing selectivity.[6,35] In addition, the increased lipophilicity of these inhibitors may result in a better distribution of these compounds into the gastrointestinal lipid phase which would translate into an improvement in their oral bioavailability. This improvement may be mandatory to compensate for the drop in antiplatelet and anticoagulant potency of the neutral boronates compared with the very potent, nonselective boroarginine derivatives.[6,7] The increased selectivity of the peptide boronates with unchanged side chains is probably due to the more hydrophobic primary specificity pocket of thrombin. The increase in selectivity, however, was accompanied by a decrease in the k_{ass} value of the uncharged methoxypropyl boronate with thrombin. Consequently, higher concentrations of this inhibitor were required to inhibit thrombin-induced platelet aggregation.[6] It seems probable that the negative electrostatic potential around thrombin's active site increases the rate of interaction with positively charged molecules[3] and, thus, the uncharged boronates would react more slowly with thrombin. It remains to be determined whether it is possible to design rapidly reacting boronates with charged P_1 residues.

In parallel with the development of mechanism-based inhibitors, other groups have developed active-site inhibitors that do not rely on an interaction with the catalytic residues to achieve tight binding. Intensive screening of a group of arylsulfonylarginine amides led to the identification of Argatroban as selectivc inhibitor of thrombin with a K_i in the nanomolar range.[31] NAPAP was selected from a group of benzamidine based compounds as a selective and potent inhibitor of thrombin.[37] The crystal structures of complexes with Argatroban and NAPAP demonstrate that they use the same interaction areas within the active site of thrombin as those used by PPACK. Their mode of binding is, however, quite different. In contrast to PPACK, they bind in a compact U-shaped conformation. The basic residue (arginine or benzamidine) is bound to the primary specificity pocket while the hydrophobic groups on either side fold together and occupy the S_2 and aryl-binding pockets.[38,39] Both Argatroban and NAPAP display a kinetic profile that closely matches the one for an ideal inhibitor outlined in Table 1; they are both highly selective for thrombin and exhibit k_{ass} values with thrombin greater than 10^7 $M^{-1}s^{-1}$ (ref. 6,7). Argatroban is effective in preventing

thrombosis in various animal models[40] and has recently been licensed in Japan for use in various thrombotic indications.

Exosite inhibitors

C-terminal fragments of hirudin were found to inhibit thrombin's cleavage of fibrinogen without inhibiting its activity with low molecular substrates that bind only to the active site.[41] The crystal structures of thrombin-hirudin complexes demonstrated that this was due to their binding at the anion-binding exosite which blocked the necessary interaction of fibrinogen with this site.[42] However, the affinity of these fragments for thrombin was only in the micronolar range. While this affinity could be improved by modification of acidic and hydrophobic residues in the sequence,[43,44] the K_i values obtained were somewhat higher than the 10 nM required for an ideal thrombin inhibitor. Because these peptides bind to an exosite that is unique to thrombin, they are absolutely specific for thrombin. Moreover, they react rapidly with thrombin with k_{ass} values greater than 10^8 $M^{-1}s^{-1}$ (ref. 21). Thus, their kinetic profile is ideal apart from their affinity being somewhat too low. As a consequence of their low affinity exosite-binding inhibitors have largely been supplanted by bivalent inhibitors which bind to the active site and have proved more effective in preventing thrombosis in animal models.[45]

A completely different approach to the development of thrombin inhibitors has been described by Bock *et al.*[46] DNA aptamers that inhibited thrombin were selected from a library of 10^{13} molecules. A 15mer with the sequence GGTTGGTGTGGTTGG was found to inhibit fibrinogen cleavage with a nanomolar K_i. Subsequent crystallographic analysis illustrated that this inhibition was due to exosite-binding.[47] Thus, the kinetic profile of the aptamer is expected to correspond to those of the peptide exosite inhibitors but with improved affinity for thrombin. The DNA aptamer has shown encouraging antithrombotic effects in animal models.[48]

Bivalent inhibitors

This class of compounds binds to both the active site and exosite of thrombin. The most thoroughly studied members of this class are hirudin and hirulog. Crystal structures of both these compounds in complex with thrombin have been determined.[8,9,42,49] Although both compounds bind to the anion-binding exosite in a similar manner, their interaction with the active site is markedly different. Hirudin binds to the active site in an unexpected orientation; the direction of the polypeptide chain within the active site is exactly opposite to that expected for substrates. Within the active site, however, the interaction areas utilized by hirudin overlap with those of other inhibitors. The first and third residues of hirudin occupy the S_2 and S_3 pockets, respectively. Unlike other inhibitors, however, hirudin does not bind to the primary specificity pocket.[8,9] Hirulog is a 19 residue peptide with an exosite-binding sequence based on hirudin linked by a polyglycine spacer to D-Phe-Pro-Arg-Pro which binds the active site.[50] The kinetic profiles of both hirudin and hirulog are ideal. They both react rapidly with thrombin ($k_{ass} > 10^8$ $M^{-1}s^{-1}$) to form tight complexes.[20,21] The unique mode of active-site binding by hirudin results in an absolute specificity for thrombin; hirudin is not known to

inhibit any other proteases. Although the more conventional active-site binding of hirulog will not result in absolute specificity, its exosite binding should contribute to a great increase in its specificity for thrombin over plasmin and APC. Animal studies have confirmed the antithrombotic efficacy of hirudin and hirulog in a wide range of models of venous and arterial thrombosis.[11]

THROMBIN INHIBITORS AS ANTITHROMBOTIC AGENTS

Clinical trials have investigated the use of direct thrombin inhibitors as antithrombotics in situations where the efficacy of heparin is limited. The best studied inhibitors to date are hirudin and hirulog and the major indications that have been examined are unstable angina, coronary thrombolysis and coronary angioplasty. The results of the clinical trials with direct thrombin inhibitors in the treatment of unstable angina have been encouraging. Both hirudin and hirulog appear superior to an equivalent anticoagulant dose of heparin; hirudin reduced the size of coronary thrombi and produced a decrease in subsequent myocardial infarction[51] while hirulog reduced the occurrence of unfavourable events such as death, myocardial infarction and bleeding.[52] Anticoagulants are given during and after the administration of the thrombolytic agent to accelerate the rate of recanalization and to decrease the incidence of reocclusion. In contrast to heparin, which has only a limited ability to accelerate thrombolysis, direct thrombin inhibitors have proved effective in thrombolytic therapy. In comparison with heparin, both hirulog and hirudin increased coronary patency following treatment with a thrombolytic agent.[53,54] Although heparin is routinely used in coronary angioplasty, acute reocclusion occurs in up to 10% of cases. Thus, it is encouraging that both hirudin and hirulog have been found to be effective in reducing the incidence of reocclusion.[55,56]

In most cases, the superiority of direct thrombin inhibitors can be attributed to their ability to inhibit thrombin bound to fibrin or the extracellular matrix. In coronary thrombolysis and unstable angina, thrombin bound to fibrin or fibrin fragments is probably one of the major causes of rethrombosis and direct thrombin inhibitors should, thus, be more effective in preventing rethrombosis than heparin.[11] In addition, coronary angioplasty leads to the exposure of the sub-endothelial extracellular matrix and thrombin bound to this matrix also appears to be resistant to antithrombin.[14] The other major advantage of direct thrombin inhibitors over heparin is the reproducibility of their anticoagulant response. In contrast to the variable dose-response observed with heparin, all thrombin inhibitors which have been examined in detail yield a very predictable anticoagulant response for a particular dose. This predictable dose response for the direct thrombin inhibitors will obviate the need for the intensive laboratory monitoring that is necessary in the case of heparin.[11]

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