Direct Thrombin Inhibitors in Cardiovascular Medicine

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Abstract Currently used antithrombotics such as heparin have a number of potential limitations that may be overcome by the new class of agents that directly inhibit thrombin. These agents variously block the active catalytic and/or the anion binding exosites of the thrombin molecule and are potent and specific inhibitors of thrombin’s many biological actions, as demonstrated by in vitro and animal models of thrombosis. Preliminary data indicate that the direct antithrombins are safe and efficacious in humans, and their use in acute coronary syndromes and coronary angioplasty in place of heparin has yielded promising early results. Phase III trials in these clinical settings are currently under way. Newer antithrombotics that inhibit thrombin generation and thrombin activity at various strategic points within the coagulation cascade are also in the early stages of development. (Circulation. 1994;90:1522-1536.)

Key Words antithrombotics • thrombosis • coagulation • current perspectives

The introduction of the direct thrombin inhibitors—a new class of agents that specifically and potently antagonize the actions of thrombin—has opened up new and exciting opportunities in the regulation of the thrombotic process, which is so important in the field of cardiovascular medicine. It is now firmly established that coronary artery thrombosis is causal in the development of acute coronary syndromes. Thrombin in turn plays a pivotal role in both the platelet activation and the fibrin generation inherent in this process and offers an ideal target for modulation of the clinical course of these syndromes. Heparin, currently the mainstay of antithrombotic therapy, has a number of limitations that may be overcome by specific characteristics of the newer thrombin inhibitors. This review will focus on these agents, from their biochemistry through to the current status of their clinical use in the cardiological fields of acute coronary syndromes and coronary angioplasty. Their use has been evaluated in several other areas, including diseminated intravascular coagulation, hemodialysis, microsurgery, and deep venous thrombosis, but will not be covered in this review.

Limitations of Current Antithrombotics
The principal antithrombotic action of heparin depends completely on the presence of cofactors. Inactivation of thrombin: activated factor X (factor Xa); factors XII, XI, and IX; and tissue factor VIIa complex is achieved through the activation and modulation of antithrombin III. Heparin may also act by potentiating heparin cofactor II–mediated inactivation of thrombin, especially at higher doses. The therapeutic use of heparin mandates intensive and meticulous laboratory monitoring of its anticoagulant effect because of the marked individual variation in its anticoagulant response. Heparin can be inactivated by platelet factor 4 and heparinase, both of which are released by activated platelets, and fibrin monomers have been found to protect thrombin from inactivation by the heparin–antithrombin III complex in thrombogenic states. Binding to vitronectin, fibronectin, and other plasma proteins limits the amount of heparin available and can decrease its anticoagulant effect, whereas heparin therapy is ineffective in people with hereditary antithrombin III deficiency. Most important, however, is the inability of the heparin–antithrombin III complex to inactivate thrombin already bound to clot. This is most likely due to a conformational change in the thrombin molecule induced by its binding to fibrin, which, in turn, prevents attachment of the heparin–antithrombin III complex. The “protected status” of clot-bound thrombin is a major potential drawback to heparin use, as this thrombin can act as an ongoing source of thrombogenesis at sites of pathological thrombus formation, theoretically limiting heparin efficacy. In contrast, the actions of direct thrombin inhibitors are antithrombin III independent; they do not bind plasma proteins to a significant extent, are able to inactivate clot-bound thrombin, and prevent thrombin-induced platelet activation, thus offering several potential advantages over heparin. Their mechanisms of action and likely therapeutic benefit in cardiovascular disorders may be better understood by reviewing the biology of thrombin itself.

Biology of Thrombin
Thrombin, the key regulator of the thrombotic process, is a glycosylated, trypsinlike serine protease, with lysine- or arginine-directed specificity. It is generated from prothrombin by the prothrombinase complex—factors Xa, V, calcium, and phospholipids, with factor Xa cleaving the prothrombin molecule to form the A
Protein A

Prothrombin

Factor Xa

VIIIa

Platelet Aggregation

Fibrinogen

Protein S

Activated Protein C

Inactivation Va, VIIIa

and B chains of thrombin, which combine to form α-thrombin.

Thrombin has many and varied biologic functions, but its main action is to catalyze the transformation of fibrinogen to fibrin, whether the thrombin is soluble in plasma or fibrin bound. Thrombin further activates factor XIII to cross-link fibrin and stabilize the clot, and promotes and amplifies clot formation by activating other clotting plasma proteins, including factors V and VIII. Of importance, it also is one of the most potent agonists for platelet recruitment and aggregation, which further reinforces the newly formed clot. Thrombin also initiates a series of counterregulatory reactions to trigger endogenous anticoagulant systems to maintain homeostasis. Through its binding to thrombomodulin, thrombin activates protein C, which joins with protein S to inactivate factors Va and VIIIa (Fig 1). It also stimulates the release of both tissue-type plasminogen activator (TPA) and plasminogen activator inhibitor type 1, its natural antagonist, to further regulate endogenous thrombolysis.

There are complex interactions between the endothelium and thrombin, with thrombin promoting vasodilation where the endothelium is intact, through release of prostacyclin and nitric oxide, but causing vasoconstriction where endothelium is damaged, through liberation of endothelin. Thrombin also acts as an effector molecule, occupying receptors on the endothelium and smooth muscle cells to activate cellular proliferation, as well as stimulate the release of platelet-derived growth factor (PDGF). Leukocyte and monocyte chemotaxis and release of interleukin-1 from macrophages are also induced by thrombin.

The unique structure of thrombin is believed to account for its high specificity for its substrates. The location of the catalytic binding site in a deep narrow canyon on the thrombin molecule surface appears to restrict access to other macromolecules by steric hindrance. Thrombin, distinct from most other serine proteases, has an anion-binding exosite, also known as the substrate recognition site, that is separate but adjacent to the active catalytic site. Fibrinogen binds at this site, increasing thrombin’s specificity for it. Thrombomodulin and hirudin also attach here, whereas heparin appears to have its own basic binding site. Direct thrombin inhibitors known to block the anion-binding site have been shown to inactivate thrombin without displacing the thrombin molecule from the fibrin surface, providing evidence that thrombin has a further and separate binding site for fibrin distinct from its fibrinogen binding site.

The apolar binding site is located in a hydrophobic pocket close to the catalytic site in the fibrinopeptide groove and is also involved in substrate binding to the active catalytic site, as well as thrombin attachment to the platelet receptor glycoprotein 1b. The main platelet receptor for thrombin, however, was recently characterized by Coughlin and colleagues and has the unique feature of a tethered ligand. After thrombin binds the receptor through a hirudin-like anion-binding exosite, it cleaves the receptor to reveal a new receptor’s amino acid terminus, which functions as the tethered ligand to activate the receptor. The molecule of thrombin thus remains free to activate other receptor sites and propagate the thrombotic process.

The regulation of thrombin is complex, involving proteinase inhibitors antithrombin III, heparin cofactor II, and α2-macroglobulin. Thrombin generation is controlled through coagulation amplification via factors including V and VIII; interaction with thrombomodulin, protein C, and protein S; as well as partitioning in the clot itself.

Direct Thrombin Inhibitors

History

The therapeutic use of the leech (Hirudo medicinalis) probably dates back to the ancient Greeks, but it was Haycraft who first described the antithrombotic prop-
Hirudin was isolated by Markwardt in the 1950s, and the structure has subsequently been characterized and cloned. Further development based on the structure of hirudin has led to the formation of a family of hirudin-like peptides called hirugen or hirulog. Peptide analogues hirugen and hirulog are based on the structure of hirudin, and synthetic derivatives argatroban, D-phenylalanyl-L-prolyl-L-arginyl chloromethylketone (PPACK), and the boroarginine derivatives have subsequently been developed.

**Structure and Function**

**Hirudin**

The prototypic thrombin inhibitor is the naturally occurring hirudin, isolated from the leech saliva, and is the most potent and specific known inhibitor of thrombin, forming a tight, highly stable noncovalent complex with thrombin. It is a 65-amino-acid protein with three disulfide bridges and a molecular weight of ≈7000 d. Multiple isoforms of natural hirudin exist, with slightly different numbers of amino acids but similar anticoagulant activities. All have the disulfide bridges and a sulfated tyrosine residue in position 63.

Recombinant DNA technology has allowed the development of recombinant hirudin (r-hirudin), which has an identical amino acid sequence to natural hirudin, with or without the sulfated Tyr. The nonsulfated molecule results in about a 10-fold reduction in thrombin affinity, although binding still remains strong. Several companies have now developed proprietary versions of r-hirudin, which are at various stages of clinical testing. Although direct head-to-head comparisons of these different recombinant have not been performed, Longstaff and colleagues, in a study designed to investigate standardization of laboratory hirudin assays, found that four proprietary formulations of r-hirudin all had similar potencies, using a simple, standardized chromogenic assay.

The hirudin molecule has two distinct domains—an NH₂-terminal core domain and a COOH-terminal tail. The N-terminal binds and inhibits the active catalytic site of thrombin, whereas the carboxy terminal simultaneously blocks the anion-binding exosite. This interaction takes place in two steps, with an initial ionic interaction and then a rearrangement of the hirudin-thrombin complex to form a stronger binding. The apolar-binding site also appears to be involved in hirudin binding, since the compound proflavin (which binds to the apolar-binding site) is displaced during formation of the hirudin-thrombin complex. Crystal structure studies have further demonstrated that the attachment of hirudin to thrombin is not limited to the three binding sites but also forms multiple other contacts, tightly binding thrombin over an extended area of the molecule. Overall, this allows hirudin to be uniquely specific for thrombin, with no inhibitory action on any other serine protease.

**Hirugen**

Hirugen, a synthetic hirudin derivative, is a dodecapeptide comprising the terminal 12 residues of hirudin involved in anion-binding exosite blockade. The binding of hirugen to thrombin is similar to the C-terminal of hirudin, and the molecule contains a sulfated tyrosine to increase its thrombin affinity. Hirugen was designed to block the interaction of thrombin with fibrinogen, leaving the catalytic site of thrombin free to interact with antithrombin III, enhancing its actions, with or without heparin. However, the in vivo antithrombotic activity of hirugen is considerably weaker compared with hirudin and hirulog, and it has not progressed to testing in the clinical arena.

Fig 2. Schematic of the thrombin molecule, illustrating its key binding sites. Thrombin interactions with the direct thrombin inhibitors hirudin, hirulog, hirugen, PPACK, and argatroban are illustrated.
Hirulog

A group of peptides called hirulogs were subsequently developed that had the D-Phe-Pro-Arg sequence of the N-terminal of hirudin linked to the hirugen molecule by a segment of glycy1 residues varying from 6 to 18 Å in length.40 Like hirudin, this synthetic derivative is able to block both the active catalytic site and anion-binding site of the thrombin molecule (Fig 2). Hirulog-1 has four glycy1 residues between the two domains and has been found to be a potent thrombin inhibitor.40 Although hirulog retains the high specificity of hirudin for thrombin, its substrate-type binding to thrombin is more typical of other protease inhibitors, in contrast to the unique multiple contact binding of hirudin.36 However, doubt has arisen regarding the contribution of active site inhibition to hirulog’s action, following the discovery that thrombin itself mediates slow cleavage of hirulog at the Arg-Pro bond on the amino-terminal extension in vivo,41 effectively converting it into a hirugen-like molecule. This may account for some of the pharmacological differences with hirudin40 and has led to the development of hirudin derivatives with noncleavable bonds.42

PPACK and Argatroban

PPACK is a tripeptide synthetic compound that has a structure very close to fibrinopeptide A.43 It contains the amino acid sequence corresponding to the cleavage site of fibrinogen and acts as an affinity agent to thrombin.44 PPACK blocks the active catalytic site by alkylating the active site histidine residue and is an irreversible inhibitor of thrombin (Fig 2). The compound has been found to be an effective thrombin inhibitor in plasma and in vivo, but the presence of a reactive group leads to a relatively rapid loss of activity due to reactions with other plasma components.45 Argatroban, an arginine derivative, binds thrombin at the apolar-binding site adjacent to the active site. Its main action is to block thrombin’s active catalytic site as does PPACK, but unlike this compound, argatroban is a competitive antagonist. Comparative studies with other thrombin inhibitors have shown this compound to be a potent thrombin inhibitor with strong affinity for thrombin.44,46

Other Thrombin Inhibitors

Many other compounds have been developed that have antithrombin activity, but are too toxic for clinical use (Table 1). Thrombin inhibition by arginine and benzamidine derivatives ranges from weak to strong,44,45 whereas the borogargin derivatives such as DuP 714 are potent antithrombins and have the potential for oral bioavailability.47 However, to date use has been limited by adverse liver toxicity, believed to be related to the boron constituent.

Several other classes of antithrombins, at various stages of development, are aimed at controlling the thrombotic process at different strategic points or through novel methods. The low molecular weight heparin (LMWH) preparations are fragments of standard heparin obtained by chemical or enzymatic depolymerization of the polysaccharide chains. Although they are antithrombin III dependent and cannot inhibit thrombin that is fibrin bound, they are differentiated from heparin by both their relative and absolute increased affinity for factor Xa over thrombin,48 with the degree of selectivity for factor Xa varying with different commercial preparations. The LMWH compounds have lower affinities for endothelial cells and plasma proteins than standard heparin and are therefore cleared more slowly, allowing once-daily dosing and more predictable anticoagulant responses.48 Several trials have now demonstrated their clinical use, particularly in the areas of deep venous thrombosis prophylaxis and treatment.49

A novel mechanism for control of the thrombotic process has been developed using single-stranded DNA oligonucleotides known as aptamers to bind target proteins and block their actions. Thrombin aptamers, first described by Bock and colleagues,50 bind the fibrinogen recognition site on the thrombin molecule51 and have demonstrated potent antithrombotic effects in vitro,50 in animal models of thrombosis,52,53 and also have the ability to inhibit clot-bound thrombin.53 The 15 nucleotide aptamer used in a number of studies has the distinct characteristics of rapid onset of action and short half-life, which may favor its future use in certain acute clinical settings.52,53

Alternative strategies for controlling thrombin activity are becoming available through the isolation and

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**Table 1. Classification of Antithrombotic Agents**

<table>
<thead>
<tr>
<th>Classification of Antithrombotic Agents</th>
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<td>Argatroban</td>
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<td>Thrombin aptamers</td>
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<td>Thrombin generation inhibitors</td>
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<td>Factor X inhibitors</td>
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<td>Tick anticoagulant peptide</td>
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<td>Vitamin K antagonists</td>
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<tr>
<td>Warfarin sodium</td>
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development of agents that selectively inhibit key enzymes at different points within the coagulation cascade. From a theoretical standpoint, because the direct thrombin inhibitors antagonize thrombin that is preformed, some thrombin activity may still occur before its neutralization. New formation of thrombin remains unaffected and may also contribute to a “leakage” of thrombin activity despite the presence of an inhibitor. Conversely, inhibition of activated factor X (factor Xa) can prevent new formation of thrombin and disrupt the thrombin feedback loop that autoamplifies thrombin generation but has no effect on thrombin that is already present. Inhibition of proteins earlier in the pathway, such as tissue factor—a key initiator of thrombosis in vivo—offers a more mechanistically specific targeting for prevention of thrombosis but may have limited efficacy because of compensatory augmentation in activity of alternate coagulation pathways such as the intrinsic pathway.

With these theoretical considerations in mind, a number of thrombin generation inhibitors have undergone early experimental testing. Local inhibition of factor Xa has been achieved by antistasin, a 119-amino-acid protein initially isolated from the salivary gland of the Mexican leech,54 and tick anticoagulant peptide (TAP) derived from the soft tick,55 now produced by recombinant technology. Both are potent and highly selective antagonists of factor Xa.55,56 and unlike anti-thrombin III, can inhibit factor Xa attached to the prothrombinase complex as well as free factor Xa.56. When used as adjuncts to thrombolysis, these agents, through the inhibition of thrombin generation, accelerated clot lysis and prevented reocclusion.57,58 This has brought into focus the controversial concept that, at least in part, it is the continuous generation of new thrombin rather than reexposure of preformed clot-bound thrombin that is responsible for the phenomenon of reocclusion. Indeed, markers of thrombin generation such as prothrombin fragment F1.2 have been found to increase during and after thrombolytic treatment for acute myocardial infarction, suggesting that increased thrombin activity associated with thrombolysis is, at least in part, due to new thrombin generation.59 This in turn may have important implications for the eventual place of direct thrombin inhibitors as adjuncts to thrombolysis, as lack of inhibition of new thrombin generation represents a potential mechanistic limitation of these agents.

Recombinant forms of naturally occurring endogenous anticoagulants is yet another strategy that has been exploited to control the thrombotic process. Tissue factor pathway inhibitor (TFPI) is a multivalent proteinase-inhibitor, whose complex actions involve the binding and inhibition of factor Xa, producing a factor Xa–TFPI complex that causes feedback inhibition on the factor VIIa–tissue factor complex responsible for triggering factor X activation.60,61 Purified and recombinant activated Protein C, through their inhibition of factors Va and VIIa, inhibit thrombin formation62 and have also been found to inactivate plasminogen activator inhibitor.63 In vitro and animal studies have demonstrated antithrombotic effects of this agent,63,64 and human evaluation in phase I trials has already commenced.65 A potential use for this compound is as an adjunct to thrombolysis, particularly following the discovery that thrombolytic therapy itself appears to generate activated protein C.66 Novel approaches such as monoclonal antibodies against or peptidomimetics of factor VII and antibodies against the recently characterized platelet thrombin receptor are all in the very early stages of development.

Experimental Studies With Direct Thrombin Inhibitors

As expected from their mode of action, direct thrombin inhibitors antagonize the broad spectrum of thrombin’s actions. Hirudin has been shown to inhibit fibrin formation; prevent activation of factors V, VIII, and XIII; and prevent thrombin-mediated platelet activation in vitro.67,68 It produces virtual total inhibition of thrombus formation ex vivo in both rat and human plasma69 and may also augment displacement of factor Xa from vascular endothelium, actually associating itself with factor Xa.69 Animal studies have confirmed the antithrombotic efficacy of hirudin in a wide range of animal venous and arterial models of thrombosis,27,30,70-73 Hirulog,64-66 argatroban,65,67 and PPACK74 have also proven to be effective antithrombotics in various animal models. Hirudin appears to have additional effects on thrombus composed predominantly of platelets rather than fibrin,77 although in a rat disseminated intravascular coagulation model higher concentrations of hirudin were required to inhibit platelet deposition than to prevent fibrin deposition.79

Direct thrombin inhibitors may have an especially important adjunctive role in thrombolysis. Apart from their main action to activate plasmin and lyse fibrin, thrombolytics also trigger a number of reactions that engender a paradoxically “prothrombotic state,” including plasmin-mediated activation of factor XII and platelet activation,80 platelet release of plasminogen activator inhibitor,81 and consumption of plasma plasminogen, which in turn draws clot-bound plasminogen into the soluble phase, reducing the substrate for activation and attenuating fibrinolytic efficacy (plasminogen steal).82 Of chief significance, however, is that lytic agents accelerate exposure of thrombin bound to fibrin within the clot, as well as increase the amount of free thrombin,83·85 producing a milieu highly conducive to rethrombosis. Experimental models both in vitro and in vivo have demonstrated the ability of thrombin inhibitors to neutralize both clot-bound thrombin and free soluble thrombin.12,86-88 Hirudin has been shown to accelerate thrombolysis following TPA,89 and prevent reocclusion in a canine model,90 whereas hirulog accelerated thrombolysis and prevented reocclusion in a rat model.74 Argatroban, an agent with less affinity for thrombin than either hirudin or hirulog, was able to accelerate clot lysis by TPA in a canine stenotic coronary artery model but required the addition of aspirin to reduce the incidence of reocclusion.91

There is further compelling experimental evidence to support a beneficial role for direct thrombin inhibitors in the field of coronary intervention. Platelet aggregation and fibrin deposition produced in a pig model of carotid angioplasty have been successfully prevented by hirudin,78 and the same agent also inhibited restenosis at 28 days in a rabbit angioplasty model, suggesting an additional mechanism of inhibition of thrombin’s effector molecule function as a stimulator of smooth muscle.
Thrombin inhibitors effectively prevented thrombin-induced platelet activation and comparative studies with heparin found hirudin to be more effective in reducing both platelet and fibrin deposition on coronary stents, platelet thrombi in the porcine deep arterial injury model, and more potently inhibited thrombus in rabbit and rat thrombosis models.

**Clinical Pharmacology and Phase I Studies**

Relatively little data are available from comparative and systematic pharmacological trials, although hirudin remains the most extensively studied thrombin inhibitor pharmacologically. Recombinant hirudin is poorly absorbed orally and is administered parenterally via the intravenous, intramuscular, or subcutaneous route. Primate studies have demonstrated faster onset and earlier return to baseline after intravenous administration. The half-life after intravenous use was 40 minutes compared with almost 2 hours after subcutaneous injection. The volume of distribution has variously been estimated from 8.9 to 17.2 L with significant distribution into the extravascular space. Little hepatic metabolism takes place with hirudin, and 95% of the compound is renally excreted in its active form. Impaired elimination has been demonstrated in patients with renal insufficiency, and dose reduction is recommended in patients with renal dysfunction.

In a recent pharmacological trial with human volunteers, hirulog differed from hirudin in that its half-life was 15 to 20 minutes shorter after intravenous bolus administration and that total body clearance was more rapid at 0.5 L/min for a 70-kg man compared with hirudin’s clearance rate of 0.19 L/min. However, only 20% of hirulog was recovered from the urine, suggesting that it undergoes more extensive metabolic clearance than hirudin, including hepatic metabolism or proteolysis at other sites.

Several phase I trials have now been completed with the various thrombin inhibitors, demonstrating excellent safety and anticoagulant efficacy profiles in healthy volunteers. Argatroban was well tolerated in healthy human volunteers and produced a dose-dependent prolongation of the coagulation parameters activated partial thromboplastin time (aPTT) and thrombin time (TT), with a return to baseline levels 1 hour after intravenous administration. No prolongation of skin bleeding time was detected, even with concomitant administration of aspirin.

The biologic effects of sulfated r-hirudin (CGP-39393) were comprehensively studied in a recent report by Verstraete and colleagues. Hirudin was administered subcutaneously, as either a single dose or repeated doses, in 231 healthy volunteers. The drug was extremely well tolerated, with no significant adverse events reported. Single injections of 0.1 mg/kg prolonged the aPTT to almost twice baseline values in 184 volunteers, and increasing doses up to 0.75 mg/kg increased the aPTT linearly. The anticoagulant effect after subcutaneous administration was noted within 30 minutes, peaking at 4 to 6 hours, and returning to baseline within 24 hours. Similar findings have been reported elsewhere. Repeated subcutaneous doses lengthened aPTT for as long as the administration was continued, with no evidence of cumulative effects even up to 6 days of administration. Bleeding time was not prolonged even up to doses of 0.5 mg/kg, suggesting little effect of hirudin on primary hemostasis.

Tolerability and anticoagulant activity of hirulog were assessed in a recent randomized, placebo-controlled study of 54 healthy volunteers, with both intravenous and subcutaneous routes of administration studied. Doses ranged from 0.05 to 1.0 mg/kg, and a rapid dose-dependent prolongation of the aPTT for both intravenous and subcutaneous routes was demonstrated. There was no significant bleeding or other adverse events reported, and overall there was no prolongation of the skin bleeding time. Subcutaneous injection resulted in a later peaking of anticoagulant effect and a sustained prolongation of aPTT over several hours. Greater anticoagulant activity was achieved at lower doses of hirulog by extending the intravenous infusion period for 2 hours or more, after steady-state levels of the drug were obtained.

**Clinical (Phase II) Studies**

Several studies have assessed the role of thrombin inhibitors in acute ischemic syndromes and PTCA (Table 2).

**Acute Myocardial Infarction**

A number of small studies published recently have assessed thrombin inhibitors as adjuncts to thrombolysis. Lidon et al randomized 42 patients to heparin or hirulog after streptokinase for acute myocardial infarction. Hirulog had significantly better 90-minute Thrombolysis in Myocardial Infarction (TIMI)-3 patency rates (61% versus 29%, P = .05) and 120-minute patency rates (75% versus 36%; P < .02). Bleeding complications were the same in the two groups. Tabata et al evaluated argatroban in the prevention of reocclusion in 24 patients following successful reperfusion therapy for myocardial infarction. Reocclusion at 1 month was 0% compared with 15% in 74 patients on heparin control. This effect was independent of the type of reperfusion therapy or residual stenosis after treatment.

Results of the Hirudin for the Improvement of Thrombolysis (HIT) study were recently published. In this dose-escalation study, three doses of recombinant hirudin (HBW 023) were used as adjunctive therapy following front-loaded TPA for myocardial infarction in 143 patients. Patency rates were determined at 30, 60, and 90 minutes; at 36 to 48 hours; and after discharge. Ninety-minute TIMI-3 patency rates increased, and reocclusion rates diminished with increasing doses of hirudin. This trial did not have a heparin control arm, but patency rates compared favorably with those previously established for heparin, and the reocclusion rates were lower than the 10% to 15% rate associated with adjunctive heparin therapy. There were three spontaneous hemorrhages in the entire study group, and an increase in puncture site bleeding was noted in the highest dose group (5 of 83 patients).

Cannon and colleagues reported the findings of the TIMI-5 trial that randomized 246 patients to either hirudin or heparin for 5 days following accelerated TPA for myocardial infarction. There was a trend for improved patency at 90 minutes in the hirudin group (64.8% versus 57.1%), whereas TIMI-2 or -3 flow at 18 to 36 hours was significantly improved with hirudin
TABLE 2. Recent and Ongoing Trials With Direct Thrombin Inhibitors

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<th>Year</th>
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<th>Condition</th>
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<td>Randomized</td>
<td>Angiographic</td>
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<tr>
<td>HIT\textsuperscript{106}</td>
<td>1993</td>
<td>Hirudin</td>
<td>143</td>
<td>MI</td>
<td>Observational</td>
<td>Angiographic</td>
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<tr>
<td>TIMI-5\textsuperscript{107}</td>
<td>1994</td>
<td>Hirudin</td>
<td>260</td>
<td>MI</td>
<td>Randomized</td>
<td>Angiographic</td>
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<tr>
<td>TIMI-6\textsuperscript{110}</td>
<td>1994</td>
<td>Hirudin</td>
<td>193</td>
<td>MI</td>
<td>Randomized</td>
<td>Death, MI</td>
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<td>TIMI-8</td>
<td>NA</td>
<td>Hirulog</td>
<td>Planned</td>
<td>UAP</td>
<td>Randomized</td>
<td>Clinical efficacy</td>
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<tr>
<td>TIMI-9</td>
<td>NA</td>
<td>Hirudin</td>
<td>3000 planned*</td>
<td>MI</td>
<td>Randomized</td>
<td>Mortality</td>
</tr>
<tr>
<td>GUSTO-2</td>
<td>NA</td>
<td>Hirudin</td>
<td>12 000 planned*</td>
<td>MI and UAP</td>
<td>Randomized</td>
<td>Mortality</td>
</tr>
<tr>
<td>OASIS</td>
<td>NA</td>
<td>Hirudin</td>
<td>10 000 planned</td>
<td>UAP or MI without ST elevation</td>
<td>Randomized</td>
<td>MI</td>
</tr>
<tr>
<td>HIT-II</td>
<td>1993</td>
<td>Hirudin</td>
<td>7000 planned†</td>
<td>MI</td>
<td>Randomized</td>
<td>Mortality and reinfarction</td>
</tr>
<tr>
<td>HELVETICA</td>
<td>1994</td>
<td>Hirudin</td>
<td>1154</td>
<td>PTCA</td>
<td>Randomized</td>
<td>Early complications restenosis</td>
</tr>
<tr>
<td>Hirulog in PTCA</td>
<td>1994</td>
<td>Hirulog</td>
<td>4600</td>
<td>PTCA</td>
<td>Randomized</td>
<td>Abrupt closure</td>
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</tbody>
</table>

TIMI indicates Thrombolysis In Myocardial Infarction; HIT, Hirudin for Improvement of Thrombolysis; GUSTO, Global Use of Strategies to Open Occluded Arteries; OASIS, Organization to Assess Strategies for Ischemic Syndromes; HELVETICA, Hirudin in a European Restenosis Prevention Trial Versus Heparin Treatment in PTCA Patients; NA, not available; PTCA, percutaneous transluminal coronary angioplasty; UAP, unstable angina pectoris; and MI, myocardial infarction.

*Trials stopped prematurely and restarted because of excessive bleeding events with heparin and hirudin.
†Terminated in July 1994 because of excessive bleeding with hirudin.
Adapted from Theroux and Lidon\textsuperscript{114} with permission.

(97.8% versus 89.2%, \( P = .01 \)). There were less reclosures (1.6% versus 6.7%, \( P = .07 \)) and a lower incidence of death or recurrent myocardial infarction during hospitalization in the hirudin group (6.8% versus 16.7%, \( P = .02 \)) (Fig 3). However, it is worth noting that the heparin control group in this study had a much higher event rate than has been found in other and much larger studies.\textsuperscript{108,109} No difference was found in bleeding rates between the two groups. Data from the TIMI-6 study, evaluating adjunctive hirudin versus heparin with streptokinase following myocardial infarction, revealed a trend toward lower event rates in the hirudin group, using a composite end point of death, reinfarction, new congestive heart failure, and shock (Fig 4).\textsuperscript{110} These data are very encouraging and have laid the foundation for large-scale evaluation by prospective, randomized trials.

Unstable Angina

A number of small-scale trials have provided optimistic results with the use of direct thrombin inhibitors in unstable angina. Sharma et al\textsuperscript{111} assessed the safety and efficacy of hirulog in 20 patients with unstable angina using a composite end point of death, myocardial infarction, intractable angina, presence of intracoronary thrombus, and bleeding and found that a 5-day hirulog infusion resulted in a more favorable outcome than in 51 heparin controls. Lidon et al\textsuperscript{112} used three escalating doses of hirulog in 55 patients with unstable angina. Fifteen patients were involved in a short-term dose-escalating protocol, whereas the remaining 40 patients received a 72-hour infusion of hirulog at one of three escalating doses. Anginal symptoms were controlled in 35 of the 40 patients. Four of the 5 clinical failures occurred in patients on the low or intermediate dose of
Lefkovits and Topol

Direct Thrombin Inhibitors

Fig 3. Bar graphs of results of the TIMI-5 trial evaluating hirudin versus heparin as adjunctive therapy to accelerated tissue-type plasminogen activator for acute myocardial infarction. Improved patency, less reocclusion, and greater "late reopening" were found with hirudin. Adapted from Cannon et al.\textsuperscript{107} with permission.

Hirulog, and there were no deaths, infarctions, or bleeding complications.

The recently released results of the TIMI-7 trial evaluating hirulog in unstable angina have expanded on the potential beneficial role of direct thrombin inhibitors in acute coronary syndromes.\textsuperscript{113} Four hundred ten patients were included in a double-blind, dose-ranging study design in which hirulog was administered as a 72-hour infusion at one of four escalating doses. Although the combination of the three higher dose groups did not reduce the incidence of the primary end point of an unsatisfactory outcome (death, myocardial infarction, recurrent ischemic pain with ECG changes, or rapid clinical deterioration within 72 hours) compared with the lowest-dose group, there was a significant reduction in rates of death or nonfatal myocardial infarction at the time of hospital discharge and at 6 weeks in the combination group.\textsuperscript{113} However, there was no heparin control group.

The thrombin active site inhibitor argatroban was administered in an escalating-dose regimen by Gold and colleagues\textsuperscript{114} in 43 patients with unstable angina. There

Fig 4. Bar graphs of results of the TIMI-5 trial of hirudin versus heparin with accelerated tissue-type plasminogen activator, and the TIMI-6 trial of hirudin versus heparin following streptokinase for acute myocardial infarction. A, Comparison of the composite end point of death or reinfarction for the hirudin and heparin groups in the TIMI-5 trial. B, Comparison of the composite end point of death, reinfarction, new congestive heart failure, or shock for the heparin and the three hirudin groups in the TIMI-6 trial.
was a dose-dependent increase in aPTT and a decrease in fibrinopeptide A levels with argatroban administration and no angina during the infusion period. However, there was recurrence of angina in 9 of the 43 patients (21%) at an average of 5.8 hours after the infusion was terminated. Early recurrent angina correlated with a higher argatroban dose and a greater prolongation of aPTT. There was a rise in thrombin–antithrombin complex over baseline levels after drug cessation, as well as a return of fibrinopeptide A levels to baseline levels, suggesting that rebound thrombin generation may be associated with the early recurrence of angina. This phenomenon of “rebound” unstable angina is discussed more fully.

Topol and colleagues recently completed a large-scale trial of a thrombin inhibitor in unstable angina. One hundred sixty-three patients with rest pain, abnormal ECG, and visual presence of thrombus in the culprit lesion >60% were randomized to one of four escalating doses of hirudin or heparin control. The heparin group was divided into either conventional aPTT range (65 to 90 seconds) or high aPTT range (90 to 110 seconds), and efficacy end points were angiographic indexes of the culprit vessel. Patients treated with hirudin tended to show greater improvement in minimal cross-sectional area (P = .028), average cross-sectional area (P = .08), minimal luminal diameter (P = .029), and percent diameter stenosis (P = .07) (Fig 5). Hirudin prolongation of aPTT was comparable to the heparin control group in the high-aPTT range, and its incremental benefit over both heparin groups suggests that the in vivo actions of hirudin may not be truly reflected by its in vitro “anticoagulant” effect. There were seven major bleeds, all procedure related, and three spontaneous bleeds, none of which required transfusion. Although it was not the primary end point, the study also found a trend toward a decrease in subsequent myocardial infarction in the hirudin group (1.7% versus 8%, P = .11).

PTCA

Both hirudin and hirulog have been found to be safe and effective antithrombotics in patients undergoing diagnostic coronary angiography. Topol et al recently demonstrated for the first time that coronary angioplasty can be performed safely and effectively with an anticoagulant other than heparin. In a multicenter trial, 291 patients pretreated with aspirin were given one of five escalating doses of hirulog instead of heparin during elective coronary angioplasty. The infusion was continued for 4 hours, and the primary end point was abrupt vessel closure within 24 hours of the procedure. Acute closure occurred in 11.3% of patients receiving the lower three dose regimens and in 3.9% of the patients receiving the higher two dose regimens (P = .052). There was a rapid and dose-dependent rise in aPTT and activated clotting time (ACT), with abrupt closure occurring in only three patients whose ACT was >300 seconds. One patient had a significant bleeding complication. The study concluded, that at the very least, thrombin inhibitors warrant further development in the area of thrombosis inhibition during coronary intervention.

Hirudin has also been used during PTCA in 113 patients by Van den Bos et al. In a 2:1 randomized design, 74 patients received hirudin and 39 patients received heparin at the time of electively for stable angina. The infusions were continued for 24 hours, and doses were adjusted to aPTT levels. There was a trend for fewer periprocedural events (myocardial infarction and bypass surgery) in the hirudin group (1% versus 10%) and a significant reduction in ischemic events based on ST changes >0.1 mV during 24-hour Holter monitoring (4% versus 14%). There were four bleeding episodes in the hirudin group, but these all occurred at puncture sites.

Phase III Trials

The encouraging results from the phase II studies have ushered in a number of large multicenter trials evaluating the role of direct thrombin inhibitors that are either under way or reaching the final stages of planning. The Global Use of Strategies to Open Occluded Arteries (GUSTO-II) trial is currently enrolling 12 000 patients in a trial of hirudin versus heparin in addition to standard management in acute coronary syndromes. Patients who present within 12 hours of symptoms of cardiac ischemia at rest with ischemic ECG changes (ST elevation, ST depression, or definite T-wave inversion) are eligible, regardless of whether they are classified as having unstable angina or non-Q-wave or Q-wave myocardial infarction. The study medication is infused for 3 to 5 days, and the primary end point is a composite of death and reinfarction.

The Canadian multicenter Organization to Assess Strategies for Ischemic Syndromes (OASIS) trial will focus on patients in the early phase of unstable angina and myocardial infarction without ST elevation. In a 2×2 factorial design, the effects of a single regimen of hirudin versus heparin and warfarin versus placebo will be assessed, with study end points including cardiovascular death, myocardial infarction, and refractory angina. Enrollment of 8000 to 10 000 patients is planned. The TIMI-8 trial will examine the use of hirulog in unstable angina, and the role of hirudin as an adjunct to thrombolysis will be assessed in the TIMI-9 and European HIT-II trials. The TIMI-9 study plans to recruit ~3000 patients, whereas HIT-II has a planned sample size of 7000 patients. Expected completion of all these large-scale studies is within 12 to 18 months.

![Bar graph of results after 3 to 5 days of hirudin or heparin in unstable angina, summarizing the angiographic findings for overall treatment groups. Reproduced from Topol et al118 with permission.](http://circ.ahajournals.org/)

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**Fig 5.** Bar graph of results after 3 to 5 days of hirudin or heparin in unstable angina, summarizing the angiographic findings for overall treatment groups. Reproduced from Topol et al118 with permission.
The role of direct thrombin inhibition in PTCA is being evaluated in a large-scale European multicenter trial known as the Hirudin in a European Restenosis Prevention Trial Versus Heparin Treatment in PTCA Patients (HELVETICA). Enrollment of nearly 1200 patients with unstable angina in this randomized, heparin-controlled study of hirudin in coronary angioplasty is now complete, with early complications and angiographic restenosis at 6 months the major efficacy end points. Results will be available late in 1994. In this trial, hirudin was given intravenously for only 24 hours and then subcutaneously for an additional 48 hours in the most aggressive arm. If the study's findings are negative, it will still not fully resolve the question of whether more sustained infusions of hirudin could optimize the potential benefit of hirudin. A second trial, assessing hirulog use in 4600 patients during PTCA, is also under way, but this has only short-term phase end points.

Emerging Issues in Clinical Use of Direct Thrombin Inhibitors

Laboratory Measurement of Antithrombotic Activity

The introduction of the direct thrombin inhibitors into the clinical arena has raised the issue of the most appropriate laboratory measure to monitor these agents' activity. Focus has predominantly been on the global clotting assays such as aPTT, prothrombin time (PT), TT, and ACT, although more recently the ACT and TT have been found to be relatively unsuitable for monitoring these agents, leaving aPTT as the marker most commonly used. In general, direct thrombin inhibitors have potent in vitro anticoagulant effects, prolonging aPTT, PT, and ACT in a dose-dependent fashion, although the PT assay, as with heparin, appears to be a much less sensitive marker of these agents' anticoagulant actions. The in vitro anticoagulant effect of these agents has been found to be highly consistent and reproducible, in contrast to heparin, which produces variable responses among patients and requires meticulous and individual patient adjustment (Fig 6).

Despite this, the diagnostic value of aPTT with direct thrombin inhibitor use remains questionable. Increasing doses of antithrombin can have contradictory effects on aPTT. Although a number of studies have found aPTT to be insensitive at high or very low doses of r-hirudin, Talbot found that hirudin could be used over a wide range of doses and still result in a controllable increase of aPTT. Different aPTT reagents and methods may also yield different in vitro results. Of note, in patients receiving sufficient antithrombotic therapy to raise the aPTT to > 60 seconds, the portable Biotrack 512 Coagulation Monitor (CIBA-Corning Diagnostics) was found to result in an approximately 10-second longer aPTT value than conventional hospital laboratory methods (GUSTO-1, unpublished data).

Furthermore, although aPTT correlates well with the clinical antithrombotic effect of heparin, this relation was determined empirically, and the same therapeutic ranges may not hold true for the direct thrombin inhibitors. Antithrombotic doses of the direct thrombins that are equally potent with heparin appear to have much less prolongation of aPTT in experimental models and in a human phase I trial with hirulog. Conversely, fibrinogen depletion and, to a lesser extent, fibrin degradation products resulting from thrombolysis prolong aPTT, yet this occurs in the presence of heightened thrombin activity known to be associated with lytic therapy.

There may also be differences in aPTT response among the various thrombin inhibitors themselves. A comparison of the aPTT response to hirudin and hirulog from two large multicenter trials revealed that although there was incremental prolongation of aPTT with increasing doses of hirulog, the aPTT plateaued at the higher doses of hirudin (Fig 7). Nevertheless, both trials showed clinical efficacy of the thrombin inhibitor, leaving the significance of the difference in dose responses of aPTT uncertain and the issue of the appropriateness of aPTT as a marker for antithrombotic effect unresolved. Appropriate laboratory ranges of aPTT and other in vitro anticoagulant tests should therefore be clearly established in large-scale trials, evaluating these agents over wide dosage ranges and in varied clinical settings, before their widespread application in the clinical setting.

Alternative laboratory evaluation of thrombin inhibitors may be achieved with more complex chromogenic and immunological tests that quantitate the direct action of hirudin on thrombin, but these tests do not give a functional assessment like the clot-based assays. Newer assays such as fibrinopeptide A (released during the cleavage of fibrinogen by thrombin) and prothrombin fragment (F1.2) (generated on conversion of prothrombin to thrombin) are direct markers of thrombin activity and have been found to decrease in a dose-dependent fashion after thrombin inhibitor administration. Small studies have already used F1.2 with intracoronary stents as a marker for antithrombotic adequacy and predictor of adverse outcomes, although results are conflicting. These assays are still not routinely or quickly available to guide therapy, and their eventual use requires more comprehensive evaluation before they are accepted as the preferred laboratory measures.
Lack of a Dose-Response Curve

Data from the completed phase II trials reviewed above has delineated a striking and remarkably consistent trend regarding direct antithrombin dose and clinical response. Although these studies demonstrated clinical benefit with direct antithrombin use, no trial to date has demonstrated any clear-cut dose-response curve of biologic or clinical efficacy for the direct thrombin inhibitors. In the TIMI-5 and TIMI-6 studies evaluating hirudin as an adjunct to thrombolysis, escalating doses of hirudin were used, from a bolus of 0.15 mg/kg and a 0.05 mg/kg per hour infusion to a bolus of 0.6 mg/kg and a 0.2 mg/kg per hour infusion. Clinical benefit was found in all except the lowest-dose group, yet increasing the dose of the agent did not incrementally improve study outcomes.\(^{107,110}\) Similarly, in the study of Topol and colleagues\(^ {115}\) of hirudin in unstable angina, comparable doses of hirudin were used, but no differences in clinical efficacy were found among the three highest doses. This “paradox” of clinical antithrombotic benefit without a clear-cut dose-response effect was found in the HIT study of hirudin in myocardial infarction\(^ {106}\) and the TIMI-7 trial of hirulog in unstable angina\(^ {113}\) and occurred with hirulog use with angioplasty as well, as found by Topol and colleagues.\(^ {118}\)

The reasons for this lack of evident dose-response remain uncertain, yet there are a number of possible explanations. Although these studies, involving approximately 150 to 300 patients each, may simply be underpowered to demonstrate clinical differences among the various dose groups, these findings possibly represent saturation of thrombin-binding sites or, in other words, a “low-threshold” phenomenon for efficacy of these agents. Their clinical effect may be achieved at relatively low doses, with further dose increases unable to further improve clinical outcome. Alternatively, higher doses of direct thrombin inhibitors may paradoxically antagonize their own antithrombotic effect through mechanisms such as interference with the thrombin–thrombomodulin interaction, partially inhibiting activated protein C formation. The correct ratio of bolus to infusion dose remains to be determined empirically, with the finding of a lack of a dose-response potentially representing comparisons of different bolus-infusion combinations, or suboptimally proportioned bolus-to-infusion dose ratios. Whatever the explanation, the observation is noteworthy and concerning in that the issue of appropriate dosing is a crucial one, with the aim being to optimize clinical benefit with a minimum of adverse effects—principally bleeding—which is discussed below.

Bleeding Risks

Although theoretically the bleeding risk with direct thrombin inhibitors is lower than that for other antithrombicides because of its mono-target specificity, absence of direct platelet effects, and short half-life, bleeding remains the most concerning adverse effect. Phase I studies have provided support for the safety and low bleeding risk of these agents. Fox et al\(^ {108}\) did not report any significant bleeding in a study of 54 healthy volunteers, and overall, there was no prolongation of the skin bleeding time, suggesting minimal disruption of primary hemostasis. Similar findings have been reported with hirudin\(^ {101}\) and argatroban.\(^ {100}\) In a phase II study involving hirulog, major bleeding rates were acceptably low (0.34%), although minor bleeds did occur in 25%.\(^ {118}\) In the TIMI-5 trial, which combined hirudin with TPA, there was no difference in the rate of major hemorrhages between the hirudin group and the heparin controls (17.5% with hirudin versus 23.3% with heparin, \(P=NS\)). However, the vast majority of these bleeds in both groups were related to an instrumented site, and major spontaneous hemorrhage was actually lower in the hirudin group (1.2% versus 4.7%, \(P=.09\)).\(^ {107}\) Similarly, there was no excess bleeding with the higher doses of hirudin in the TIMI-6 study.\(^ {110}\)

The pilot study of hirudin in unstable angina found the occurrence of major and minor bleeding episodes to be relatively infrequent (4.4% and 1.8%, respectively), yet a potentially important relation between bolus dose and subsequent bleeding risk was demonstrated. The high bolus dose of hirudin (0.9 mg/kg) was associated with significant bleeding and required discontinuation,
implicating the bolus of hirudin as an important potential determinant of bleeding complications.115

Overall, the early experience had suggested that direct thrombin inhibitors may have acceptable bleeding rate profiles both in the normal population and in patients with coronary artery disease. In large-scale trials, however, a much wider spectrum of patients is enrolled, and the experience is greatly amplified in the numbers treated. In GUSTO-II and in TIMI-9, the dose of hirudin was substantially reduced after the start of these trials (from a bolus of 0.6 mg/kg and a 0.2 mg/kg per hour infusion to a bolus of 0.1 mg/kg and a 0.1 mg/kg per hour infusion) because of an observed excess in hemorrhagic strokes in patients simultaneously receiving thrombolytic therapy. This experience demonstrates the potent potential for serious bleeding complications at high doses and the need for large-scale controlled trials to further assess their effects.

The “Rebound” Phenomenon

Gold and colleagues114 highlighted the potential impact of rebound procoagulation that may occur after withdrawal of thrombin inhibitors.114 In their study of argatroban use in patients with unstable angina, fibrinopeptide A levels fell with the administration of argatroban, but thrombin–antithrombin III complex levels did not, suggesting argatroban may inhibit thrombin activity (formation of fibrin from fibrinogen) but does not stop ongoing thrombin generation. After argatroban was stopped, thrombin–antithrombin III complex levels rose almost fourfold above baseline values, whereas fibrinopeptide A returned to pretreatment levels. This “rebound” increase in thrombin formation and activity, in turn, was associated with recurrent episodes of angina in 23% of the patients, suggesting a possible causal relation. A follow-up study by the same group129 found the prothrombin activation fragment (F1.2) from the same patients also steadily increased during treatment and continued to rise after drug withdrawal, confirming ongoing prothrombin activation during and after argatroban infusion. Similar persistence of thrombin activation has also been demonstrated with hirudin treatment in patients with unstable angina.130

However, Willerson et al131 suggested that this rebound phenomenon may have alternate explanations. They point out that six of the nine patients in the study of Gold et al114 had heparin withdrawn within 4 hours before the argatroban infusion, which itself is associated with recurrence of angina.132 Heparin also causes increased clearance of antithrombin III, so that the rise in thrombin–antithrombin III complex levels in the study of Gold et al may merely have been a reflection of low initial thrombin–antithrombin III complex levels resulting from thrombin–antithrombin III complex clearance during the heparin infusion.131 Interestingly, no rebound angina has been noted thus far with the use of hirudin115 or hiru-log.111,112 This, in part, may be related to concomitant use of aspirin, which has been shown to have a protective effect of heparin-associated rebound.122

Costs

At present, the cost of direct thrombin inhibitors remains high, particularly in comparison to heparin, with a 3-day course of intravenous hirudin estimated to cost more than $1000. Without evidence of significant benefit over heparin from large-scale studies, the use of these agents on a routine basis cannot be advocated on clinical or economic grounds. These trials, however, are under way, and it is appropriate to limit their use at this time to study protocols and individual cases where there is a strong indication for a direct thrombin inhibitor over heparin.

Future Directions

The direct thrombin inhibitors provide potent and effective control of thrombin activity and have demonstrated usefulness and acceptable safety profiles in the cardiological fields of acute coronary syndromes and coronary angioplasty where thrombosis plays a crucial role. Yet new approaches to improve selectivity and activity of the direct thrombin inhibitors continue to evolve. A particularly promising area focuses on the interaction between thrombin and platelets. Targeting thrombin inhibitors by coupling them to Fab' portions of antibodies directed against platelet IIb/IIIa receptor or fibrin β-chain has resulted in a more potent antithrombotic than the untargeted agent in an experimental model of human thrombus,133 whereas linking direct thrombin inhibitors with platelet receptor IIb/IIIa antagonists may offer even further specificity and control over the thrombotic process.134 On the other hand, a major potential liability of direct thrombin inhibitors—lack of suppression of thrombin generation—may play out to be an important mechanistic limitation.

The introduction of the direct antithrombins has opened up a new vista in the control of the thrombotic process, but the story is far from over. We are at a critical stage, awaiting results of several large-scale trials before the widespread and routine use of these agents can be advocated, yet preliminary work has already begun with new generations of antithrombotic agents that offer even finer control of the thrombotic process at multiple and different sites within the coagulation process. The challenges ahead are considerable, and the opportunities are great, and although there is likely to be an evolution in the control of thrombosis in cardiovascular medicine, the role of thrombin inhibition will certainly be an area of intense and paramount research in the years to come.

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