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Miniperspective

Thrombin Receptor (Protease Activated Receptor-1) Antagonists as Potent Antithrombotic Agents with Strong Antiplatelet Effects

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Introduction

Cardiovascular diseases are the major cause of mortality and morbidity in the industrialized world, while their incidents are on fast ascendancy in developing countries.[1] According to the World Health Organization, 16.7 million people died worldwide of cardiovascular diseases in 2002, accounting for one-third of all deaths globally. By 2020 heart disease and stroke will become the leading cause of both death and disability in the world with the number of fatalities projected to increase to more than 20 million a year.[2,3] Approximately 40% of the 2.4 million deaths that occurred in the United States in 2002 were attributed to cardiovascular and cerebrovascular diseases with similar statistics prevailing in the European Union.[4,5] Nearly 71 million adults in the U.S. have one or more types of cardiovascular disease, resulting in a staggering cost of 403 billion dollars to the U.S. economy.[6]

Coronary artery disease (CAD[a]) is the leading cause of cardiovascular death, resulting in over half a million deaths annually in the U.S. alone.[6,7] The underlying culprit for CAD is atherosclerosis, which often has its insidious onset in early adulthood and remains undetected until symptoms manifest at a late stage.[8−10] Progressive luminal thickening due to an advancing atherosclerotic lesion often gives rise to symptoms of stable angina characterized by exertion-induced chest pain. However, most cardiovascular mortality and morbidity are associated with acute clinical manifestations of CAD usually triggered by rupture of a vulnerable atherosclerotic plaque.[11−14] The ensuing events lead to a spectrum of clinical conditions known as acute coronary syndrome (ACS) that include Q-wave myocardial infarction, non-Q-wave myocardial infarction, and unstable angina.[15−18]

Therapeutic approaches to CAD are twofold: reducing cardiovascular risk factors and treatment of ACS and related disorders.[19−22] Since the initial publication of the Framingham studies, great strides have been made in the prevention of cardiovascular disorders by reducing predisposing risk factors such as hypertension, hypercholesteremia, diabetes, and obesity.[23−25] There has been considerable education offered to the public with regard to life-style changes and smoking cessation also.[26−28] As a result of these efforts, the upsurge of cardiovascular diseases has substantially slowed.[7] Similar advances have been made in the treatment of acute coronary syndrome using innovative surgical interventions and modern pharmacological therapy.[29−32]

Antithrombotic agents are the mainstay of pharmacological therapy for acute coronary syndrome.[33,34] Mechanistically, the function of antithrombotic agents is either to prevent the formation of thrombi in the blood vessels or to dissolve the existing ones and restore blood flow.[35−38] Currently available antithrombotic agents can be classified into *anticoagulants*, *antiplatelet agents*, and *fibrinolytic agents*. The anticoagulants work either by modulating the endogenous levels of the key coagulation enzyme thrombin or by inactivating its enzymatic activity.[39−41] Antiplatelet agents inhibit platelet activation and aggregation, a key process of hemostasis and thrombus forma-

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[a] Abbreviations: ACS, acute coronary syndrome; ADP, adenosine diphosphate; APC, activated protein C; CAD, coronary artery disease; CAPRIE, clopidogrel versus aspirin in patients at risk of ischemic events; COX-1, cyclooxygenase-1; Gp, glycoprotein; ha-TRAP, high-affinity thrombin receptor activating peptide; hCASM, human coronary artery smooth muscle cells; HMW kinogen, high molecular weight kinogen; $5HT_{2A}$, 5-hydroxytryptamine receptor subtype 2A.; iv, intravenous; $P2Y_1$, purinergic P2 receptor subtype Y_1; PAR, protease activated receptor; PCI, percutaneous coronary intervention; PRP, platelet-rich plasma; TF, tissue factor; t-PA, tissue plasminogen activator; TRAP, thrombin receptor activating peptide; TxA2, thromboxane A_2; u-PA, urokinase-type plasminogen activator; VTE, venous thromboembolism.

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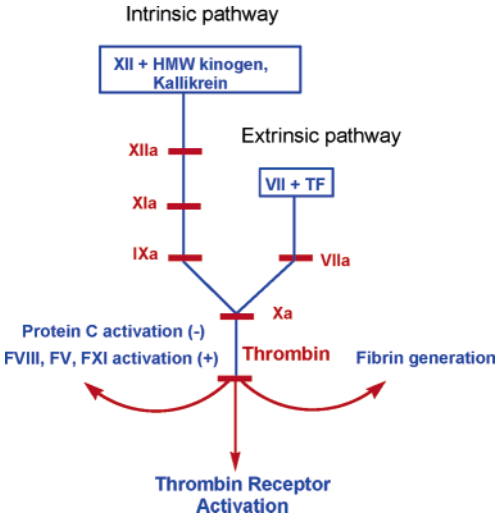


Figure 1. Simplified scheme of the coagulation cascade. The intrinsic pathway is initiated when blood comes into contact with the exposed endothelial cell surface. The extrinsic pathway is initiated upon vascular injury which leads to exposure of tissue factor (TF) to blood. The two pathways converge at the activation of factor X to Xa which, in association with the protein cofactor Va and calcium on platelet phospholipid surfaces, produces thrombin from prothrombin. In a positive feedback mechanism, thrombin activates factors VIII, V, and XI, amplifying its own signal. In a negative feedback mechanism, thrombin activates protein C. Activated protein C (APC), in combination with its cofactor protein S, inactivates the procoagulant factors Va and VIIIa, down-regulating thrombin's production. The prothrombotic activity of thrombin is mediated by proteolytic generation of fibrin and platelet activation via the thrombin receptor (PAR-1). Anticoagulants either inhibit the endogenous production of thrombin in the coagulation cascade or inhibit the catalytic activity of thrombin. See Table 1 for specific mechanisms of various antithrombotic agents: (−) negative feedback; (+) positive feedback.

tion.[42,43] Fibrinolytic agents, which are intravenously administered under clinical emergency, work by lysis of existing clots and restore blood flow in occluded vessels.[44−46]

At the center of the antithrombotic therapy is the serine protease thrombin, the main effector protease of the coagulation cascade, which is produced from its zymogen, prothrombin, by factor Xa which is the convergent common product of the extrinsic and intrinsic pathways of the coagulation cascade (Figure 1).[47−52] Thrombin is locally produced on the cell surface near its site of action upon activation of the coagulation cascade triggered by injury, and it is short-lived in plasma circulation.[47] Thrombin orchestrates a multifaceted regulatory role in the coagulation cascade by regulating its own level through the balancing of procoagulatory and anticoagulatory mechanisms. In its best known coagulatory function, thrombin proteolytically converts soluble fibrinogen to fibrin monomers, which polymerize to form an insoluble meshwork that becomes the integral part of a thrombus. Additionally, thrombin activates platelets causing them to aggregate at the site of vascular lesion contributing to thrombus formation. In fact, thrombin is the most potent platelet activator. A burgeoning thrombus, usually superimposed over a ruptured plaque, can lead to occlusive cardiovascular disorders such as unstable angina and acute myocardial infarction. In its anticoagulant role, thrombin activates the protein C system to inhibit its own production. An ideal antithrombotic agent would produce a sufficient therapeutic window between the pathophysiological role of thrombin in thrombus formation and its normal life-sustaining hemostatic functions. As discussed below, the current antithrombotic therapy falls far short of this goal.

Anticoagulants

Anticoagulants target inhibition of the enzymes of the coagulation cascade, including thrombin.[49,51,53] Depending on their mode of action, anticoagulants are classified as either *indirect thrombin inhibitors* or *direct thrombin inhibitors*.[54,55] Indirect thrombin inhibitors curtail the endogenous production of thrombin at the site of injury by inhibiting blood factors of the coagulation cascade or by activating endogenous anticoagulation mechanisms.[56,57] The best known and long used indirect thrombin inhibitors are coumarins and heparins. The coumarins inhibit vitamin K-dependent post-translational γ-carboxylation of thrombin and coagulation factors VII, IX, X, protein C, and protein S, an essential mechanism for their procoagulant properties.[58,59] The slow onset of action of coumarins and severe side effects, including bleeding, drug−drug interaction, and thrombocytopenia, often require dose titration and close patient monitoring.[60] Heparin and several of its low molecular weight analogues such as danaparoid,[61] dalteparin,[62] tinzaparin,[63] enoxaparin,[64] fondaparinux,[65] etc. with improved safety profiles are anionic polysaccharides that activate antithrombin III, an endogenous inhibitor of the serine proteases of the coagulation cascade such as factors IV, IX, X, XI, and XII.[66] Unlike coumarins, heparins have rapid onset of action but must be administered parenterally and, like coumarins, suffer from hemorrhagic side effects.[67]

Several orally active factor Xa inhibitors (Figure 2, Table 1) are currently in clinical trials as anticoagulants.[66,68−73] Factor Xa, a trypsin-like serine protease, produces thrombin from its zymogen prothrombin in the prothrombinase complex. It has been hypothesized that factor Xa inhibitors should have better safety margins than thrombin inhibitors. Because of the singularity of factor Xa as the convergent product of the coagulation cascade, a factor Xa inhibitor is expected to have a high degree of antithrombotic effect. Unless all factor Xa is inhibited, sufficient level of thrombin will be maintained for fibrin generation and other hemostatic functions, thus allowing an appreciable therapeutic window. The currently ongoing clinical trials of factor Xa inhibitors will test these hypotheses. Anticoagulants targeted to other serine proteases of the coagulation cascade such as factor VIIa, factor VIIa-tissue factor (TF), factor XIIa, and factor IXa are in various stages of preclinical research.[74]

Direct thrombin inhibitors (Figure 2, Table 1) inactivate thrombin's enzymatic activity.[75−77] The classical direct thrombin inhibitors are based on hirudin, a 65-amino acid polypeptide isolated from medicinal leeches. The recombinant hirudin analogues lepirudin and bivalirudin are widely used as antithrombotic agents.[78,79] In general, these agents suffer from lack of oral activity and short duration. There have been several efforts to achieve low molecular weight orally active thrombin inhibitors.[80,81] Ximelagatran, the first launched orally active thrombin inhibitor, has recently been withdrawn from the market because of liver toxicity.[82−84] Argatroban is another low molecular weight thrombin inhibitor that is available in intravenous formulation.[85]

Antiplatelet Agents

The second class of antithrombotic agents is antiplatelet agents.[86,87] Platelets are activated by a variety of agonists such as thrombin, ADP, thromboxane A2, epinephrine, collagen, etc. (Figure 3). Activated platelets undergo shape change and express GpIIb/IIIa receptors on their surfaces which bind to fibrinogen causing platelets to aggregate at the site of injury to form a thrombus that is further stabilized by a thrombin generated fibrin

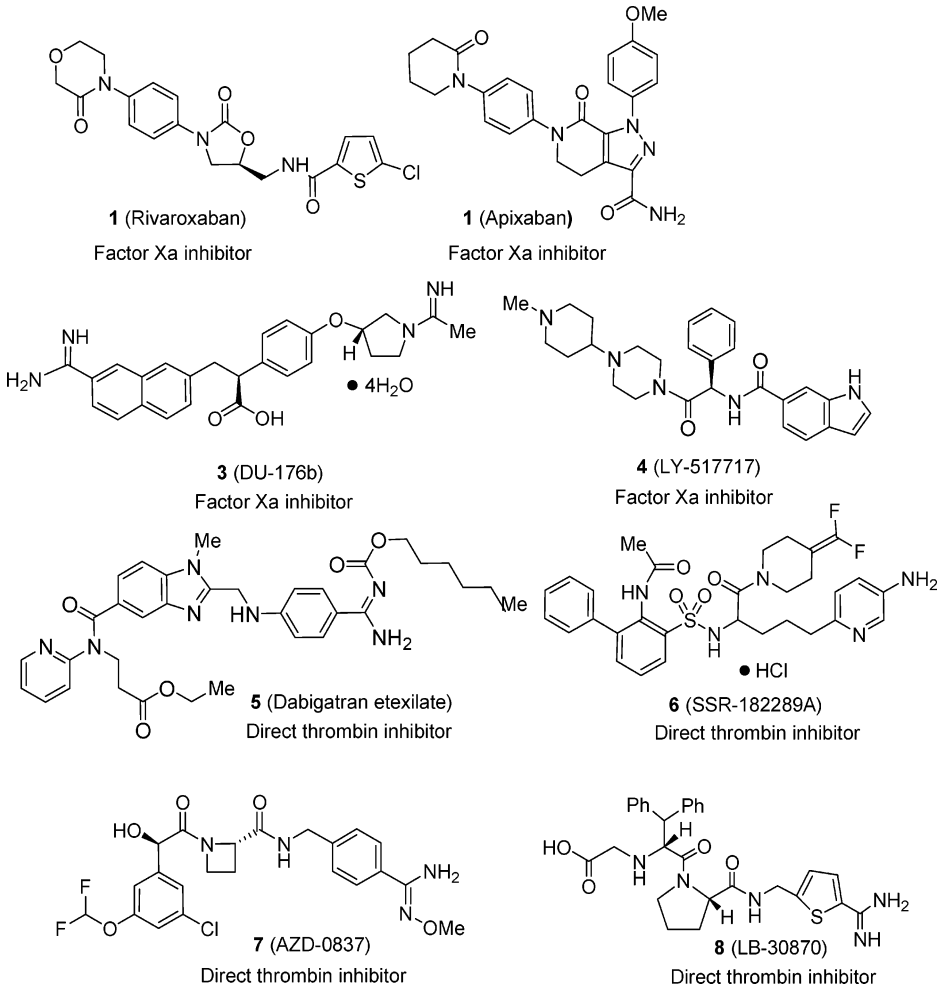


Figure 2. Examples of anticoagulants in clinical trials.

Table 1. Classification of Major Antithrombotic Agents

example	mechanism of action	status	formulation	ref
	Anticoagulants: Indirect Thrombin Inhibitors			
warfarin	vitamin K antagonist	launched	oral	58−60
heparin, danaparoid, dalteparin, tinzaparin, enoxaparin, fondaparinux	antithrombin cofactor	launched	iv or sc	61−67
1, **2**, **3**, **4** (Figure 2)	factor Xa inhibitors	clinical trial	oral	68−73
	Anticoagulants: Direct Thrombin Inhibitors			
lepirudin, bivalirudin, argatroban	inhibits enzymatic activity of thrombin	launched	iv	75−79
ximelagatran		withdrawn	oral	82−84
5, **6**, **7**, **8** (Figure 2)		clinical trial	oral	80, 81
	Antiplatelet Agents			
aspirin	COX-1 inhibition (inhibits TXA2 biosynthesis)	launched	oral	86, 87, 94, 95
abciximab, eptifibatide, tirofiban	GP IIb/IIIa receptor antagonist	launched	iv	90−92
clopidogrel, ticlopidine	$P2Y_{12}$ (ADP) receptor antagonis	launched	oral	94−98
dipyridamole	PDE inhibitor	launched	oral	99
himbacine derivative, bicyclic guanidine (see below)	thrombin receptor antagonists	clinical trial	oral	152, 194−196, 200, 201
	Fibrinolytic Agents			
streptokinase	promotes plasmin activity	launched	iv	100, 101
alteplase	promotes plasmin activity	launched	iv	100, 101
tenecteplase	promotes plasmin activity	launched	iv	105
staphylokinase	promotes plasmin activity	discovery	iv	104, 105

network.[88,89] GpIIb/IIIa antagonists such as abciximab,[90] eptifibatide,[91] and tirofiban[92] are potent antiplatelet antithrombotic agents that inhibit the end-stage processes of platelet aggregation. The currently available GpIIb/IIIa antagonists are all

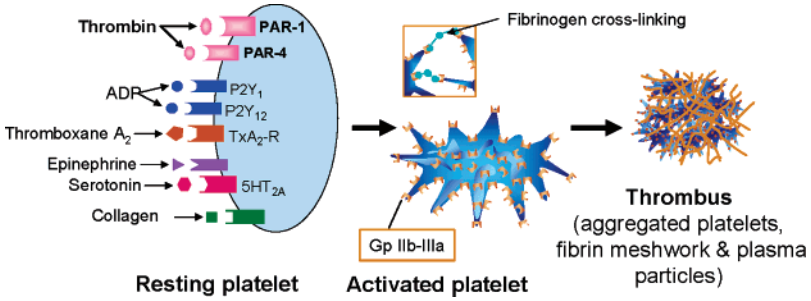


Figure 3. Thrombus formation. The coagulation pathway and platelet activation mechanisms synergize in thrombus formation. Upon endothelial injury, thrombin is locally produced. Thrombin generates fibrin from fibrinogen and activates platelets via PARs. Platelets adhere to the injured site, binding to the exposed von Willebrand factor and collagen to form an initial hemostatic plug. Activation of platelets by collagen and thrombin causes platelet shape change and release of platelet activating granular contents, which in turn amplify the platelet activation process. Activated platelets aggregate via fibrinogen cross-linking. Aggregated platelets get trapped by fibrin meshwork to form a burgeoning thrombus that further traps red blood cells and other plasma particles to form an occlusive thrombus. See footnote *a* for abbreviations.

intravenous formulations, and efforts to achieve an orally active GpIIb/IIIa antagonist have uniformly failed in clinical trials.[93]

There are several antiplatelet agents that target specific mechanisms of platelet activation.[87] Aspirin is the classical antiplatelet agent that inhibits the biosynthesis of thromboxane A-2 (TxA2), a platelet activator, by inhibiting the cyclooxygenase-1 (COX-1) enzyme. While aspirin is an inexpensive oral antiplatelet agent, its efficacy is rather low. ADP antagonists clopidogrel and ticlopidine are relatively more potent oral antiplatelet agents that inhibit ADP-induced platelet activation. The classic CAPRIE trial established the comparative benefit of clopidogrel versus aspirin for the secondary prevention of ischemic events in patients with myocardial ischemia, ischemic stroke, and peripheral arterial disease.[94,95] However, clopidogrel has only modest efficacy and it carries some risk of bleeding.[96] The combination of clopidogrel and aspirin is the current gold standard of antiplatelet therapy.[97,98] Phosphodiesterase inhibitor dipyridamole and its combination with aspirin have also been used as antiplatelet agents.[99]

Fibrinolytic Agents

Fibrinolytic agents are the primary pharmacological approach to reperfusion in the management of occlusive cardiovascular disorders that present acute clinical manifestations.[100,101] Alternative direct mechanical percutaneous coronary intervention (PCI) is a preferred approach if it can be done in a timely fashion, because of the pronounced side effects associated with fibrinolytic therapy.[102] The ease of administration of fibrinolytic agents and their universal availability make them the most widely used first-line treatment for myocardial infarction and thromboembolic disorders. Fibrinolytic agents facilitate the endogenous generation of the fibrinolytic enzyme plasmin from its proenzyme plasminogen, or they create an activated form of plasminogen with plasmin-like catalytic activity. Most marketed fibrinolytic agents are tissue-type plasminogen activator (t-PA), urokinase-type plasminogen activator (u-PA), or bacterial proteins. Fibrinolytic therapy is associated with severe shortcomings. These are short plasma half-life, induction of "paradoxical" prothrombotic condition characterized by reocclusion and systemic lysis conditions, and immunogenicity.[103] There have been several efforts to generate fibrinolytic agents with longer plasma half-life (usually in minutes) and clot selectivity with minimal systemic circulation.[104,105]

Issues Associated with Current Antithrombotic Therapy

Despite the great advances made in antithrombotic research in recent years, the currently available antithrombotic therapy suffers from several disadvantages. The major ones are side effects associated with bleeding.[106] The coagulation mechanism and platelet aggregation that antithrombotic agents target to intervene are integral to the life-sustaining normal hemostatic processes. Therefore, achieving a therapeutic window has been proven difficult. In fact, the current paradigm of antithrombotic therapy is "no bleeding, no efficacy."[107,108] Other side effects such as thrombocytopenia are also common.[109] The second issue related to the antithrombotic therapy is lack of oral efficacy. Most of the antithrombotic agents currently in use are either intravenously or subcutaneously administered. The classical anticoagulant coumadin is an oral drug, but its utility is hampered by severe bleeding, thrombocytopenia, drug−drug interactions, and idiosyncratic pharmacokinetic variations that require careful dose titrations. The first orally active direct thrombin inhibitor, ximelagatran, has been withdrawn recently from the market.[84,110,111] Among the antiplatelet agents, aspirin and clopidogrel are orally active. However, as a monotherapy or as a combination, they fall far short of the potency of GpIIb/IIIa antagonists.

There have been considerable efforts to discover potent, orally active anticoagulants and antiplatelet agents. GpIIb/IIIa antagonists are potent antiplatelets because they work by the inhibition of the end-stage mechanism of platelet aggregation. However, efforts to develop orally active GpIIb/IIIa antagonists have been unsuccessful. Therefore, there exists an unmet clinical need for a *potent*, *safe*, and *orally active* antithrombotic agent. Discussed below are the recent promising developments in the thrombin receptor (protease activated receptor-1, PAR-1) antagonist research area that have the potential to yield safe, efficacious, and orally active antithrombotic agents that work by antiplatelet mechanism. This highly promising potential is due to the fact that thrombin is the most potent platelet activator and thrombin receptor antagonism would not interfere with thrombin-mediated fibrin formation, a critical step in hemostasis.

Protease Activated Receptor (PAR)

In addition to its pivotal role in the coagulation cascade, thrombin activates various cell types such as platelets, leukocytes, endothelial cells, and vascular smooth muscle cells via proteolytic activation of specific cell-surface receptors known as *protease activated receptors* (PARs).[112−115] The prototype of these receptors is PAR-1, which is also known as the thrombin receptor.

Although a thrombin-specific receptor on platelets that mediates platelet activation has been known for some time, the exact mechanism of thrombin-specific cellular activation was unknown.[116] In 1991, Shaun Coughlin's group unveiled the intriguing mechanistic details of thrombin's cellular activation

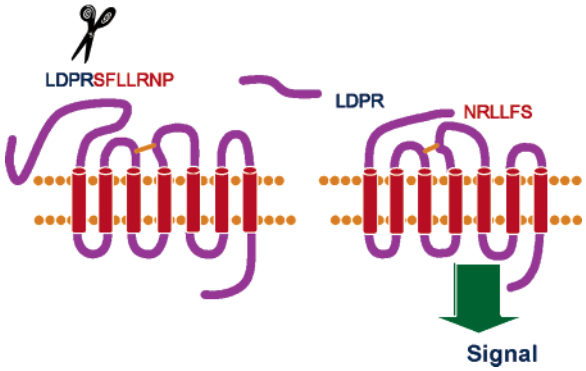


Figure 4. Coughlin's model of protease activated tethered ligand. Thrombin binds to the extracellular domain of the receptor and cleaves it at Arg41-Ser42. The newly generated N-terminus internally binds to the proximal receptor, causing cellular activation. Reprinted with permission from Macmillan Publishers Ltd.: *Nature* (http://www.nature.com) (Vu, T.-K. H.; Wheaton, V. I.; Hung, D. T.; Charo, I.; Coughlin, S. R. Domains specifying thrombin−receptor interaction. *Nature* **1991**, *353*, 674−677[122]). Copyright 1991 Nature Publishing Group, Macmillan Publishers Ltd.

by cloning of the functional thrombin receptor.[117−120] The amino acid sequence deduced from the mRNA encoding the thrombin receptor revealed a new G-protein-coupled seven-transmembrane domain receptor with a large extracellular domain.[121] The authors postulated that thrombin binds to the cellular receptor through its anion binding exosite and subsequently cleaves the extracellular domain at Arg41-Ser42 (Figure 4). The newly unmasked amino terminus acts as a "tethered ligand" binding intramolecularly to the proximal heptahelical segment eliciting G-protein-coupled transmembrane signaling.[122−125] Peptides having the sequence SFLLRN that mimic the new amino terminus of the activated receptor (known as thrombin receptor activating peptides or TRAPs) function as agonists producing functional responses such as platelet aggregation and mitogenesis.[126] Uncleavable mutant thrombin receptors failed to respond to thrombin but were responsive to TRAPs.

After the identification of the initial protease activated receptor (PAR-1), three additional protease activated receptors have been identified.[127] The current family of PAR comprises PAR-1, PAR-2,[128] PAR-3,[129] and PAR-4.[130,131] Of these, PAR-1, PAR-3, and PAR-4 are activated by thrombin. PAR-2 is activated by trypsin, tryptase, and coagulation factors VIIa and Xa but not by thrombin.[132−136] It has been established that the "tethered ligand" activation paradigm applies to all the PAR receptors. There is considerable species specificity to the nature of PAR receptors on platelets. In primates, PAR-1 is the main thrombin receptor on platelets. It is also present on other cells such as endothelial cells, smooth muscle cells, monocytes, fibroblasts, etc. PAR-4 is the second thrombin receptor on human platelets. Since PAR-4 has only weak affinity for thrombin, it is activated only at high thrombin concentrations. It is believed to be a "rescue receptor" that is activated in the event of a serious vascular lesion and the resultant high thrombin concentration. PAR-3 is found in mouse platelets where it is the major regulator of thrombin response. PAR-4 receptors are also expressed on mouse platelets. It has been recently postulated that certain PAR receptors are devoid of inherent G-protein activation but serve to function as cofactors in the activation of another PAR receptor. For example, PAR-3 serves to facilitate the cleavage of PAR-4 at low thrombin concentrations but does not become activated by thrombin.[137]

Antithrombotic Potential for a PAR-1 Antagonist

Two distinct but inter-related mechanisms are operative in the formation of a thrombus. These are activation of the coagulation cascade and activation of platelets. Thrombin, the end product of the coagulation cascade, plays a dual role in thrombosis. It cleaves fibrinogen to fibrin and activates platelets, which aggregate at the site of injury. Cleaved fibrin monomers cross-link via noncovalent interactions and the action of factor XIIIa to form an insoluble meshwork of polymerized fibrin that traps aggregated platelets and other plasma particles, allowing the thrombus to grow in size and stabilize. Depending on the site of formation, a thrombus can be either fibrin-rich or platelet-rich. Arterial thrombi, which are formed under high shear force of blood flow, are predominantly platelet-rich. Venous thrombi, which are formed under low shear stasis conditions, are fibrin-rich. It is widely believed that the high-affinity PAR-1 is more relevant to platelet activation as suggested by the following observations.[138] PAR-1 is activated at low thrombin concentrations, and antibodies to the thrombin binding extracellular domain of PAR-1 blocked this activation.[139−141] PAR-1 binds to thrombin at its anion binding exosite with high affinity. The highly specific anionic sequence that PAR-1 has on the C-terminal side of the cleavage site interacts with the fibrinogen-binding exosite of thrombin to give PAR-1 a high thrombin affinity. PAR-4 lacks such a high-affinity sequence, and therefore, it is activated only at high thrombin concentrations.[142,143] Functionally, PAR-4 may be providing some redundancy in the important platelet activation mechanism, acting as a rescue receptor in case of a severe vascular injury.

Since platelets can be activated by multiple agonists and thrombin has at least two PARs on human platelets, it is important to ask whether PAR-1 antagonism by itself is sufficient to produce substantial antithrombotic effects.[144,145] Several lines of evidence point to the fact that PAR-1 antagonism can indeed engender strong antithrombotic effects without the attendant bleeding effect that is pervasive with anticoagulants and GpIIb/IIIa antagonists. PAR-1 antagonists show functional antiplatelet activity in agonist-induced platelet aggregation measurements, Ca^{2+} transient assays, and thymidine incorporation assays.[146,147] In vivo studies carried out using synthetic PAR-1 antagonist peptides showed inhibition of platelet-rich thrombus formation in a baboon thrombosis model and a guinea pig thrombosis model, suggesting the promise of a PAR-1 antagonist for arterial thrombosis.[148,149] In another promising study, an antibody to the PAR-1 N-terminus has been reported to inhibit mechanical injury-induced thrombosis in a baboon carotid artery model without affecting bleeding time and coagulation parameters.[150,139] As described below, more recent studies using potent PAR-1 antagonists in nonhuman primate antithrombosis models have corroborated the outcome of these studies and established the therapeutic potential of PAR-1 antagonists as promising antithrombotic agents.[151−153] By selectively inhibiting the thrombin-induced platelet activation, a PAR-1 antagonist should exhibit strong antiplatelet action under conditions in which thrombin-stimulated platelet activation is critical.[148] These include acute coronary syndrome and invasive percutaneous coronary intervention (PCI) surgical procedures. Since a PAR-1 receptor antagonist is specific for the cellular actions of thrombin and does not interfere with the coagulation cascade, such agents are likely to confer an added safety margin with regard to hemorrhagic side effects, which is a complicating factor for the currently available antithrombotic therapy.

Evidence from Knockout Animal Experiments

Studies conducted using PAR deficient mice provide compelling evidence for the potential antithrombotic utility of a PAR-1 antagonist.[154] Corresponding to the human PAR-1 and PAR-4 receptors, mouse platelets contain a high-affinity PAR-3 receptor and the low-affinity PAR-4 receptor.[131] However, these receptors work in mechanistically different ways in human platelets and mouse platelets. Contrary to the human platelets where PAR-1 is the primary mediator of platelet activation, PAR-3 does not independently activate mouse platelets. Instead, it acts as a cofactor for PAR-4 mediated platelet activation.[155] Gene deletion experiments have shown that PAR-4−/− mice were totally unresponsive to even micromolar concentrations of thrombin.[155,156] On the other hand, platelets in PAR-3−/− mice could be activated by thrombin but only at high thrombin concentrations.[157] PAR-4 deficient mice were protected against thromboplastin-induced pulmonary embolism, ferric chloride induced thrombosis of mesenteric arterioles, and laser-injury induced thrombosis of cremasteric microvessels.[158−160] PAR-4 deficient mice were healthy and showed no evidence for anemia or spontaneous bleeding. Platelets in PAR-4−/− mice were normal in number and morphology, and PAR-4−/− female mice were found to be able to support pregnancy. However, as one would expect, PAR-4 deficient mice showed prolonged bleeding when challenge to hemostasis was strong.[137,160]

Studies done using PAR-3−/− mice have demonstrated that complete ablation of thrombin signaling is *not* required for protection against thrombosis. In fact, PAR-3−/− mice, which have the low-affinity PAR-4 receptors still intact, showed a level of protection against thrombosis that was similar to that seen in PAR-4 deficient mice, which showed complete ablation of thrombin mediated platelet activation.[161] Moreover, PAR-3−/− mice showed no spontaneous bleeding. The fact that PAR-3 deficient mice were protected against thrombosis suggests that even a partial attenuation of thrombin signaling in platelets might produce a therapeutically useful antithrombotic effect. Furthermore, these studies militate against the often-raised skepticism that dual PAR-1 and PAR-4 antagonism might be necessary to engender an effective antiplatelet effect. In summary, the results of functional assays and in vivo antithrombotic data of PAR-1 antagonists taken together with the gene deletion experimental data provide strong support for the antithrombotic potential for a PAR-1 antagonist.

PAR-1 Antagonists for the Treatment of Atherosclerosis and Restenosis

The pathophysiology of atherosclerosis and restenosis suggests a strong involvement of the PAR-1 receptor. In addition to its antithrombotic potential, a PAR-1 antagonist has potential utilities in the treatment of these disorders also. For example, PAR-1 is up-regulated in vascular smooth muscle cells in response to vascular injury in animal models and in atherosclerotic plaques from human arteries but not in normal arteries.[162−164] Elevated levels of thrombin were detected in arterial injury and in neointima of human atherosclerotic lesions.[165] Thrombin-mediated endothelial and smooth muscle cell activation results in the secretion of various inflammatory mediators, as well as increased vascular permeability to plasma proteins. Thrombin stimulates adhesion of neutrophils and monocytes to vascular endothelium, enhances fibroblast growth factor induced endothelial cell proliferation, and causes mitogenesis in macrophages, fibroblasts, and leukocyte and epithelial cells.[116,148,166] The thrombin receptor is expressed on smooth muscle cells and macrophages from atherectomy samples isolated from human blood vessels.[167] PAR-1 knockout mice showed complete ablation of thrombin signaling in mouse fibroblasts.[143] PAR-1 deficiency and blockade appear to show protective effects in various models of inflammation, glomerulonephritis, colitis, and restenosis in arterial injury models.[168,164]

Restenosis is the reocclusion of blood vessels in patients who have undergone invasive surgical procedures such as balloon angioplasty, endarterectomy, or stenting. Currently stenting is the preferred mode of surgical intervention, which reduces the chance of restenosis substantially.[169,170] Despite the recent promising reports of drug-coated stents, there is still an unmet clinical need for the treatment of restenosis.[171] The pathology of restenosis is characterized by extensive smooth muscle cell proliferation and remodeling or narrowing of the vessel. It has been reported that the thrombin receptor is expressed on the surface of cells in humans and baboons following angioplasty procedures.[149,172] Also, it has been demonstrated that hirudin, a potent inhibitor of the enzyme activity of thrombin, prevents angioplasty-induced smooth muscle cell proliferation in rabbits and baboons.[173,174] These data provide a mechanistic rationale for using a PAR-1 antagonist to prevent restenosis.

Since thrombin is the most potent activator of platelets, a PAR-1 antagonist should bring about strong antiplatelet effects. Additionally, the proliferative and inflammatory events that mark the underlying etiology of restenosis and atherosclerosis will be selectively inhibited at the site of the injured blood vessel.[175] This dual mode of antiplatelet and antiinflammatory−antiproliferative action makes a PAR-1 antagonist an attractive therapeutic target for the treatment of thrombosis and restenosis. Since fibrin generation will be unaffected by a PAR-1 antagonist, such an agent is likely to confer the added safety margin of low bleeding liability.

Table 2. Optimization of PAR-1 Peptide Agonists

		EC_{50}[a] (μM)
9	SFLLR-NH_2 (human sequence)	0.40
10	SF(f)LLR-NH_2	0.13
11	SF(f)F(Gn)LR-NH_2	0.04
12	SF(f)F(Gn)L-NH_2	0.28

[a] Platelet aggregation assay.

Thrombin Receptor (PAR-1) Antagonists

The early PAR-1 antagonists were designed on the basis of the sequence of the tethered ligand.[176] Functional assays such as platelet aggregation, GTPase turnover, proliferation assays using thymidine incorporation, and intracellular calcium mobilization were used to identify agonists. It was originally found that the 14 amino acid-containing peptide amide SFLLRNP-NDKYEPF-NH_2, which mimics the sequence of the N-terminal portion of the tethered ligand, was a full agonist.[177] The pentapeptide amide SFLLR-NH_2, incorporating the N-terminal sequence of the tethered ligand, was identified as the minimal structural motif required for retaining the full agonist activity.[178−180] Further optimization was achieved by substitution of positions 2 and 3 with unnatural amino acid-containing basic side chains. Substitution of phenylalanine at position 2 with *p*-fluorophenylalanine and leucine at position 3 with *p*-guanidinophenylalanine gave SF(f)F(Gn)LR-NH_2, which is the most potent pentapeptide with full agonist activity (Table 2).

Antagonists were designed from the structure of the optimized pentapeptide **11**, incorporating an early observation that certain acylations of the N-terminus would give antagonist properties.[181] The most potent antagonists were generated by replacement of the serine residue in **11** with a *trans*-cinnamoyl group. Com-

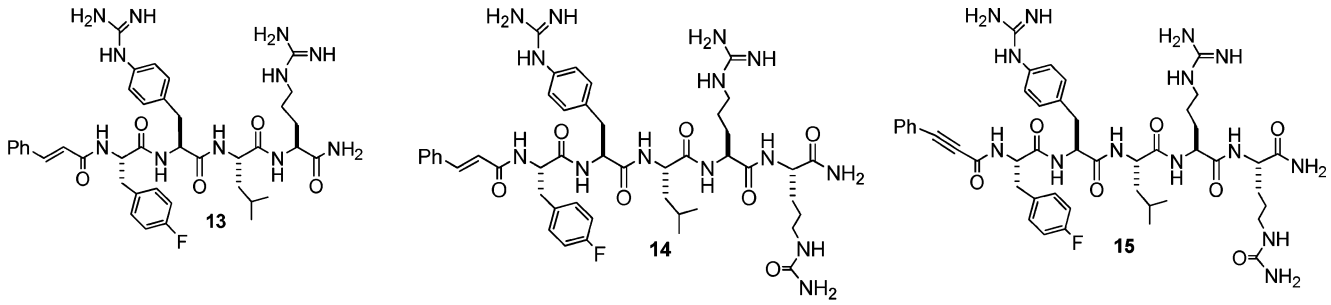


Figure 5. Peptide antagonists.

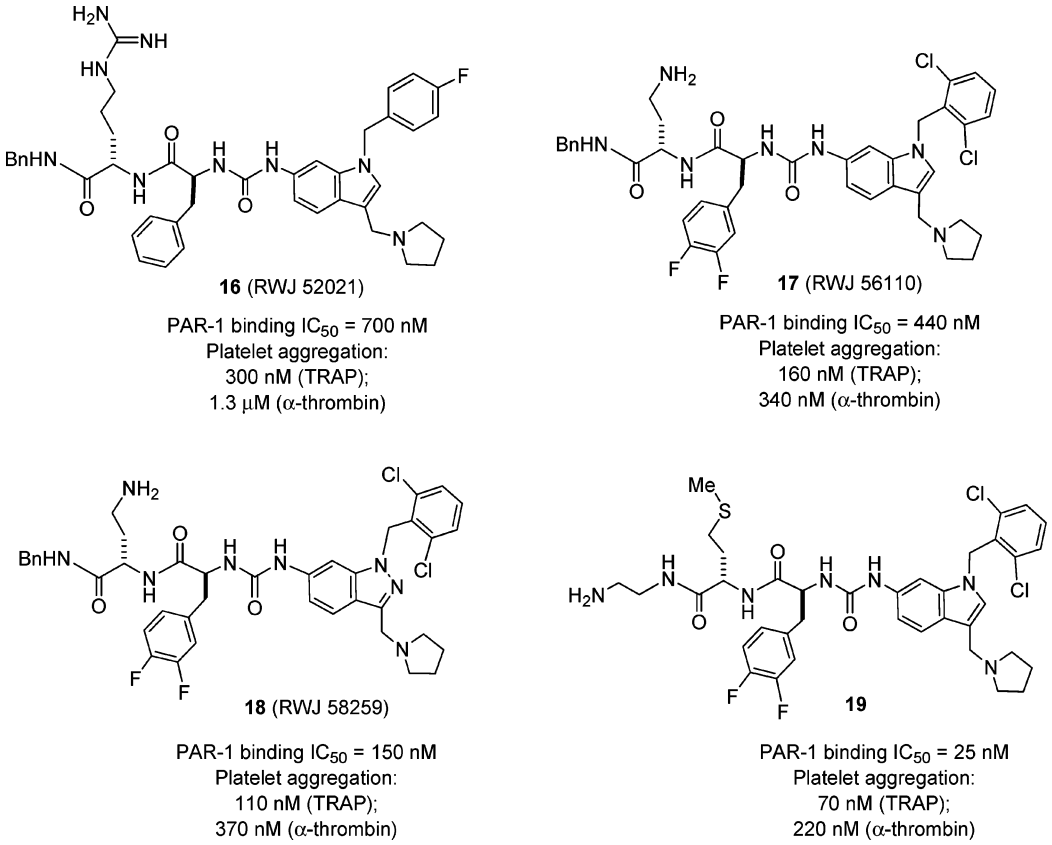


Figure 6. Peptide mimetic thrombin receptor (PAR-1) antagonists from Johnson and Johnson.

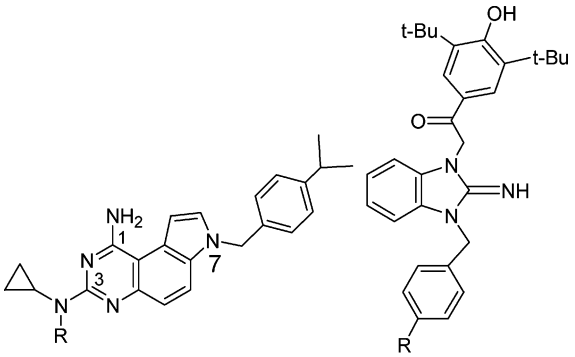
pound **13** showed an IC_{50} of 8 nM in the radioligand binding assay against the PAR-1 receptor (Figure 5). Extension of the C-terminus with a basic residue gave improved potency in the agonist-induced platelet aggregation inhibition assay. Peptide **15**, which replaces the *trans*-cinnamoyl group with a phenylpropynoyl group, was an early tight binding peptide antagonist (IC_{50} = 4 nM).[178]

Peptide Mimetic Antagonists

Peptidomimetic PAR-1 antagonists were designed on the basis of the SFLLRN motif of the tethered ligand of the PAR-1 receptor.[182,183] The essential structural requirements of the agonist peptide were established to be a free amino group at position 1, an aromatic residue such as phenylalanine at position 2, and a basic residue such as arginine at position 5. On the basis of distance parameters taken from models of SFLLRN and low-energy conformations, a three-point model relating to the distance among the amino terminus, the benzene ring of phenylalanine, and the central carbon of the arginine guanidine group was constructed. A 6-aminoindole linked peptide scaffold was constructed to spatially display the three key functional groups that formed the main scaffold of the peptide mimetic PAR-1 antagonists.

This approach initially led to the identification of PAR-1 antagonist **16** (Figure 6), which exhibited IC_{50} values of 0.7 and 0.3 μM, respectively, in the radioligand binding assay and the platelet aggregation inhibition assay using SFLLRN-NH_2 as the agonist.[182,184] Further optimization led to compounds with improved potency in both thrombin and TRAP-induced platelet aggregation inhibition assays. However, these compounds manifested unexpected hypotensive effects in the guinea pig efficacy model.

This problem was circumvented by replacing the indole group with an indazole moiety to generate an isosteric compound **18**. In the platelet aggregation inhibition assay, **18** gave IC_{50} values of 0.11 and 0.37 μM against TRAP and thrombin, respectively.



20 R = H IC_{50} = 70 nM **22** R = H IC_{50} = 65 nM

21 R = Me IC_{50} = 45 nM **23** R = Me IC_{50} = 33 nM

Figure 7. Early non-peptide thrombin receptor antagonists. IC_{50} values are shown for the receptor binding assay.

In an ex vivo guinea pig platelet aggregation model, this compound inhibited thrombin (2 U/mL) induced platelet aggregation at 0.3 mg/kg upon intravenous administration. Although this compound was inactive in two standard thrombosis models (arteriovenous shunt and Rose Bengal intravenous photoactivation assay) in guinea pig, it blocked thrombin-induced calcium mobilization and cell proliferation in the rat endothelial smooth muscle cells (which contain mainly PAR-1). More importantly, **18** showed significant reduction of neointima thickness in a rat restenosis model after perivascular administration, establishing the proof-of-concept that a thrombin receptor antagonist could have therapeutic utility in the treatment of vascular disorders such as restenosis and atherosclerosis.

The PAR-1 antagonist **18** was tested in cynomolgus monkeys in a vascular injury antithrombosis model.[151] The compound was administered intravenously, and the degree of vessel occlusion caused by electrolytic injury-induced thrombus in each carotid artery was characterized. Compound **18** significantly reduced occlusion in the vessels of all animals, which were completely occluded under experimental conditions in the absence of drug. Although the plasma level of the drug was relatively high (12 μM), ex vivo platelet aggregation measurements indicated complete PAR-1 inhibition under these conditions. In the drug-treated group, not only was the thrombus size reduced but the composition of the thrombus indicated a switch from platelet-rich thrombi to platelet-depleted thrombi, demonstrating the antiplatelet property of a PAR-1 antagonist.

Tighter binding peptidomimetic antagonists with a basic amine C-terminus have been reported recently. For example, compound **19** has a PAR-1 IC_{50} value of 25 nM in the radioligand binding assay.[185] In the TRAP-6 induced platelet aggregation assay, compound **19** showed a relatively robust IC_{50} of 70 nM. However, parallel enhancement of potency in the platelet aggregation inhibition induced by α-thrombin was not observed, which perhaps could be attributed to a fast off rate for the compound from the PAR-1 receptor. To effectively compete with the tethered ligand, a PAR-1 antagonist not only needs to be tight binding but also should have a slow dissociation from the receptor.

Non-Peptide Thrombin Receptor Antagonists

The pyrroloquinazoline analogues represented by structures **20** and **21** were the first non-peptide PAR-1 antagonists reported with good PAR-1 affinity and promising activity in functional assays (Figure 7).[186,187] These compounds showed very specific SAR. A *p*-isopropylbenzyl group at N-7, a free amino group at C-1, and a substituted amino group at C-3 were required for reasonable affinity. In the radioligand binding assay using [^{3}H]-ha-TRAP, **20** and **21** gave IC_{50} values of 70 nM (K_i = 35 nM) and 45 nM (K_i = 22 nM), respectively. Analysis of saturation binding of [^{3}H]ha-TRAP in the presence and absence of compound **20** indicated that this compound is a competitive inhibitor of PAR-1.

Compounds **20** and **21** blocked platelet aggregation induced by PAR-1 selective agonist ha-TRAP in a concentration-dependent fashion, with IC_{50} values of 300 and 150 nM, respectively. The inhibition of platelet aggregation by compounds **20** and **21** was selective, as evidenced by the fact that at 10 μM they had no effect on aggregation induced by 100 μM ADP or 5 μM collagen. Compounds **20** and **21** also inhibited aggregation induced by α-thrombin with IC_{50} values of 300 and 700 nM, respectively. In contrast to the sustained inhibition of ha-TRAP-induced aggregation, the inhibition of thrombin-induced aggregation was transient and the observed delay in aggregation was dependent on the concentration of thrombin used. At 0.5 nM thrombin, full aggregation was delayed by several minutes, whereas at 10 nM thrombin, no significant delay was seen. These compounds did not inhibit aggregation induced by PAR-4 tethered ligand peptides nor did they have any effect on platelet aggregation induced by γ-thrombin. Binding of these drugs to platelet was reversible, and full reversal of inhibition required platelets to be washed free of drug for 20 min. These compounds had no agonist activity at concentrations as high as 3 μM nor did they inhibit the catalytic activity of thrombin.

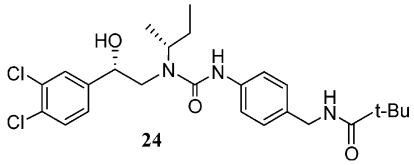
PAR-1 antagonist **20** inhibited calcium transients induced by thrombin (3 nM) and the peptide agonist TFLLRNPNDK-NH_2 (30 μM) with K_i values of 82 and 55 nM, respectively. In contrast to platelets where the inhibition of thrombin-induced effect was transient, inhibition of the calcium transients in hCASMC was sustained over the time course of the assay.

Benzimidazole derivatives **22** and **23** have been reported to be high-affinity thrombin receptor antagonists with potent ha-TRAP and thrombin-induced platelet aggregation inhibition.[188] Compound **22** inhibited ha-TRAP and thrombin-induced platelet aggregation with IC_{50} values of 265 and 600 nM, respectively. Urea and phenylisoxazole-based PAR-1 antagonists have also been reported.[189−191] These compounds displayed submicromolar IC_{50} values in the PAR-1 radioligand binding assay and a TRAP-6 induced 5-hydroxytryptamine (5-HT) secretion functional assay (Figure 8).

PAR-1 antagonists based on cyclic guanidine and amidine templates have been reported in the patent literature.[192−195] The monocyclic guanidine derivatives represented by structure **26** seem to have only modest potency against the PAR-1 receptor in the assays reported (Figure 9).

The bicyclic amidine derivatives are generally more potent. For example, compound **27** has a strong affinity for the PAR-1 receptor (IC_{50} = 17 nM), is potent in the rat smooth muscle cell proliferation assay, and has good potency in the thrombin-induced human platelet aggregation inhibition assay.

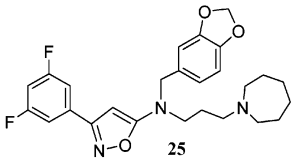
Eisai Co. has reported a PAR-1 antagonist based on the bicyclic amidine motif to be in clinical trials for acute coronary syndrome (Table 1).[196] The structure of this compound is not known with certainty but is believed to be **28** (Figure 10) on the basis of available information.[197,198] In the radioligand binding assay, this compound showed an IC_{50} of 19 nM. It inhibited TRAP-induced human and guinea pig platelet-rich plasma (PRP) aggregations with IC_{50} values of 31 and 97 nM, respectively. It also inhibited thrombin-induced human and



24

PAR-1 binding IC_{50} = 120 nM

5-HT secretion IC_{50} = 230 nM

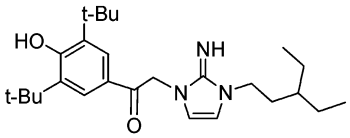


25

PAR-1 binding IC_{50} = 150 nM

5-HT secretion IC_{50} = 90 nM

Figure 8. Urea and isoxazole based thrombin receptor antagonists.

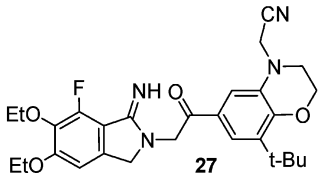


26

PAR-1 binding IC_{50} = 74 nM

Platelet aggregation (α-thrombin) IC_{50} = 540 nM

Proliferation assay IC_{50} = 300 nM



27

PAR-1 binding IC_{50} = 17 nM

Platelet aggregation (α-thrombin) IC_{50} = 290 nM

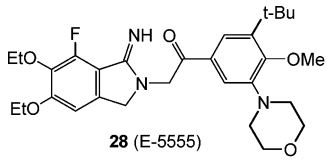
Proliferation assay IC_{50} = 6 nM

Figure 9. Guanidine and amidine based thrombin receptor antagonists.

guinea pig PRP aggregations with IC_{50} values of 64 and 130 nM, respectively. It was reported to be active in a guinea pig thrombosis model at 30 and 100 mg/kg with no change in bleeding up to 1000 mg/kg.

Himbacine-Based PAR-1 Antagonists

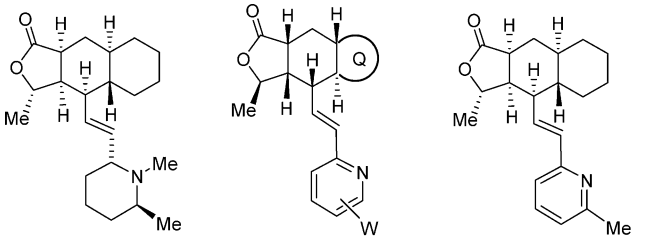
PAR-1 antagonists based on the core structure of the natural product himbacine (**29**) has been reported. The original interest in himbacine was for its antimuscarinic properties, for central nervous system indications. A total synthesis of himbacine was carried out, and several analogues were synthesized.[199,200] One racemic analogue of himbacine that has the piperidine ring system replaced by the corresponding substituted pyridine ring (**31**) was identified as a PAR-1 lead in a high-throughput assay.[153] Replacing the highly basic piperidine unit with a less basic pyridine moiety rendered this compound and the subsequently synthesized pyridine analogues inactive against the muscarinic receptors, which makes the PAR-1 antagonists selective over muscarinic receptors.



28 (E-5555)

PAR-1 binding IC_{50} = 19 nM
Platelet aggregation IC_{50}:
Human = 31 nM (TRAP); 64 nM (α -thrombin)
Guinea pig = 97 nM (TRAP); 130 nM (α-thrombin)
Active in guinea pig thrombosis model
(photochemical injury) at 30 mg/kg and 100 mg/kg
No bleeding up to 1000 mg/kg in this model

Figure 10. Presumed structure of Eisai's clinical candidate.

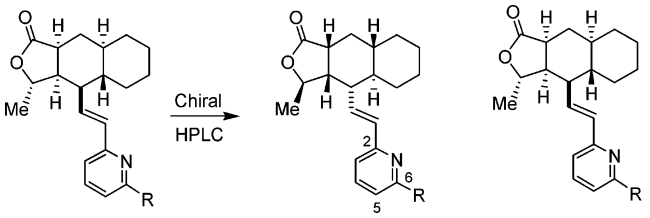


29 (+)-Himbacine (PAR-1 inactive)

30 Himbacine-based PAR-1 antagonists

31 Initial PAR-1 lead (racemic)

Figure 11. Himbacine-based PAR-1 antagonists.



Racemic

(±)-32 R = Me IC_{50} = 300 nM
(±)-33 R = Et IC_{50} = 85 nM

ent-Himbacine series

(+)-32a R = Me
IC_{50} = 150 nM
(+)-33a R = Et
IC_{50} = 20 nM

Himbacine series

(-)-32b R = Me
IC_{50} = 1500 nM
(-)-33b R = Et
IC_{50} = 400 nM

Figure 12. Enantioselective PAR-1 affinity for himbacine derivatives. The tricyclic system requires *ent*-himbacine absolute stereochemistry.

A radioligand binding assay that employed platelet membrane-bound PAR-1 receptors and radiolabeled high-affinity thrombin receptor activating peptide ([^{3}H]ha-TRAP) was used for the primary screening.[153,201] Washed human platelet aggregation induced by ha-TRAP was used as a routine functional assay. An ex vivo cynomolgus monkey platelet aggregation assay was used to study the oral activity of promising compounds. The initial lead compound **31** inhibited PAR-1 receptor with an IC_{50} of 300 nM in the radioligand binding assay (Figure 11).[152] Structure−activity relationship studies led to the identification of 6-ethyl substituted analogue **33** with an IC_{50} of 85 nM. The racemic compounds **32** and **33** (Figure 12) were resolved using chiral HPLC, and it was found that the enantiomer with absolute chirality opposite to that of himbacine (*ent*-himbacine) in the tricyclic system was more active. For example, in the radioligand binding assay **33a**, with *ent*-himbacine absolute chirality, inhibited PAR-1 binding with an IC_{50} of 20 nM whereas its enantiomer **33b** was 20 times less active. Subsequently, a number of analogues were resolved, and it was convincingly established by enantioselective synthesis that the *ent*-himbacine absolute chirality is important for PAR-1 antagonism.

Compound **33a** was evaluated in a cynomolgus monkey ex vivo platelet aggregation model after intravenous infusion (10

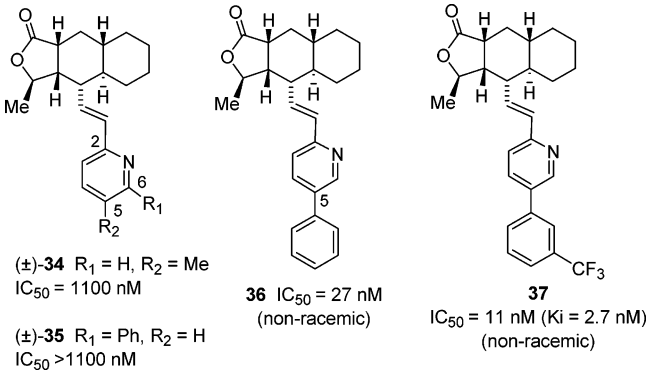


Figure 13. SAR optimization of himbacine-derived thrombin receptor antagonists.

mg/kg, 30 min). Nearly complete inhibition of platelet aggregation, induced by exogenously added peptide agonist (ha-TRAP) to the plasma drawn from the drug-treated group, was noted for 2 h. However, this compound had no oral activity, presumably because of rapid metabolism.

Although C-5 alkyl substitution and C-6 aryl substitution gave compounds with diminished PAR-1 activity (e.g., **34**, **35**), compounds with C-5 aryl substitution showed promising PAR-1 affinity (e.g., **36**) (Figure 13). Additionally, these compounds showed promising oral bioavailability. Further optimization led to C-5 phenyl derivatives carrying CF_3 and halogen substituents at the meta and ortho positions with excellent PAR-1 affinity and good oral bioavailability in a rat pharmacokinetic model.

Phenylpyridine derivative **36** showed excellent PAR-1 affinity and good oral bioavailability. A benchmark compound in the himbacine series is **37** (Figure 13).[153] This compound had a K_i of 2.7 nM against the PAR-1 receptor. It inhibited thrombin and haTRAP induced aggregation of human platelets with an IC_{50} of 44 and 24 nM, respectively. It was highly active in the thrombin-mediated calcium transient assay (K_d = 2.6 nM) and the proliferation assay (K_i = 13.0 nM) in human coronary artery smooth muscle cells. This compound showed 30% oral bioavailability in rats and 50% in monkeys. In the ex vivo platelet aggregation assay in cynomolgus monkey, **37** showed complete and sustained inhibition of platelet aggregation at 3 mg/kg after oral administration. This compound also showed potent dose-dependent inhibition of platelet deposition on thrombogenic surfaces in an arteriovenous shunt model in baboons after oral administration.[153] Compound **37** is the most potent PAR-1 antagonist reported to date.

Several variants of the himbacine tricyclic motif (Figure 14) have also been disclosed.[195] Type I compounds have the C-ring substituted with halogen atoms or functional groups such as hydroxyl, carboxylic acid, or amine groups. A type II structure has a heteroatom such as −NR−, −O−, or −S− replacing one of the C-ring carbon atoms. A type III structure has an aromatic C-ring, and the type IV structural class is devoid of a C-ring, having the B-ring substituted with lower alkyl groups. The absolute and relative stereochemical requirements for types I, II, and IV are the same, whereas these requirements are different for arylhimbacines (type III). The preferred compounds in all of these series are reported to have IC_{50} values from 4 to 100 nM and to have strong inhibition of platelet aggregation in the ex vivo cynomolgus monkey platelet aggregation model after oral administration.[201] A PAR-1 antagonist derived from the himbacine series has been reported to be in clinical trials for acute coronary syndrome.[202]

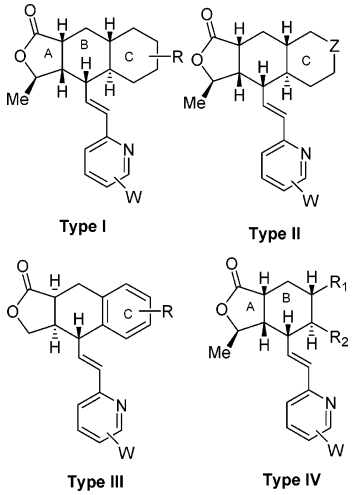


Figure 14. Different tricyclic variants of himbacine-derived PAR-1 antagonists.

Conclusion

In summary, PAR-1 antagonists hold considerable promise as antiplatelet antithrombotic agents. There exist multiple preclinical data to support this view. These include strong in vitro and ex vivo effects in platelet aggregation assays and other functional assays such as calcium transient assay and thymidine incorporation assay.[153,187] PAR-1 antagonists have shown in vivo antithrombotic efficacy in photochemical injury models in guinea pig, in a vascular injury antithrombosis model in cynomolgus monkeys, and in several oral antithrombosis models in baboons.[151,152,198] Template bleeding time remained unchanged in these studies, which suggests the promising safety margin for a PAR-1 antagonist. Knockout mouse experiments have also provided strong support for the antithrombosis potential of PAR-1 antagonists. PAR-3 knockout mice were protected against thrombosis without any change in bleeding parameters or platelet functions.[138]

There has been considerable progress in developing PAR-1antagonists as antithrombotic agents. Two pharmaceutical companies have announced that they have orally active PAR-1 antagonists in clinical trials for acute coronary syndrome.[202,203] Although PAR-1 antagonism is a promising therapeutic area, pharmaceutical research in identifying a PAR-1 antagonist has been somewhat limited. This may be because of the difficulty in obtaining a good lead or due to the perceived difficulty in finding an antagonist for the tethered ligand. A unique feature of the tethered ligand mechanism is that a high-affinity antagonist per se may not be sufficient. To effectively compete with the tethered ligand's intramolecular binding to the receptor, one needs to identify a compound with a slow dissociation rate from the receptor. This unique pharmacodynamic property adds another layer of difficulty in discovering a therapeutically useful PAR-1 antagonist. With very little information about conformation of the receptor available, one is left with a highly empirical approach. Additionally, one needs to identify an orally active compound in order to effectively compete in the antithrombotic arena. It is apparent that these difficulties have been overcome in advancing PAR-1 antagonists to clinical trials.

Thrombin is the most potent activator of platelets. Therefore, a PAR-1 antagonist should confer a potent antithrombotic effect in platelet-rich arterial thrombosis. An additional advantage of the leading PAR-1 antagonists is their oral formulation. In this regard, it is pointed out that all efforts to identify orally active GpIIb/IIIa antagonists failed in clinical trials.[93] Therefore, there

exists an unmet clinical need for a potent, safe, oral antithrombotic agent with GpIIb/IIIa antagonist-like potency and no bleeding liability. Since PAR-1 antagonism does not compromise thrombin's ability to generate fibrin and does not interfere with platelet activation mediated by other agonists, a PAR-1 antagonist is likely to have less bleeding liability in comparison to existing antithrombotic agents. Furthermore, the anti-inflammatory and antiproliferative properties of a PAR-1 antagonist should find additional utility in the treatment of atherosclerosis and restenosis. These expectations need to be validated with the help of ongoing clinical studies.

Acknowledgment. The author thanks Dr. William Greenlee for helpful discussions and Drs. Catherine Strader, John Piwinski, and Michael Graziano for their support.

Biography

Samuel Chackalamannil joined Schering-Plough in 1984 after receiving his Ph.D. from Yale University under the supervision of Prof. Samuel Danishefsky. He is currently a Distinguished Fellow at Schering-Plough Research Institute where his research has focused on cardiovascular and central nervous system drug discovery targets. His group has contributed to the discovery of several clinical candidates including a thrombin receptor antagonist based on the natural product himbacine.

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