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Review Article

Nonpeptide thrombin receptor antagonists

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Introduction

Besides the central role of thrombin in hemostasis, its cellular action was also observed by many earlier investigators (1). In 1991 two laboratories independently reported the cloning of a cellular thrombin receptor named protease activated receptor (PAR), now known as PAR1 (2, 3). This ushered in a decade of highly productive research on PARs, resulting in several thousands of papers. So far three members of thrombin receptors (PAR1, PAR3 and PAR4) and one member of trypsin/tryptase receptor (PAR2) have been discovered. PARs provide an answer to the question of how thrombin produces cellular signals. PARs are G-protein-coupled receptors that convert an extracellular proteolytic cleavage event into a transmembrane signal. Unlike most G-protein-coupled receptors, PARs carry their own ligands, which are unmasked by receptor cleavage (4). PAR1 appears to be the predominant human thrombin receptor mediating the cellular action of thrombin (5-7). Recently, much progress has been made in the development and pharmacology of PAR1 antagonists, yielding potent, selective peptidomimetic and nonpeptide antagonists. Since many excellent comprehensive reviews on PARs (5, 7-9) have been published, this review will primarily focus on recent discoveries concerning nonpeptide PAR1 antagonists, their potential therapeutic areas and PAR1 biology related to drug discovery. We will also discuss some relevant data obtained using peptide antagonists.

A family of protease activated receptors (PARs)

Table I summarizes the basic information on the family of PARs. The family of PARs presently consists of PAR1 to PAR4. The PAR1, PAR3 and PAR4 receptors are activated by thrombin (2, 3, 10, 11), whereas PAR2, whose real *in vivo* activator is not known, is activated by trypsin (12), tryptase (13) and coagulation factors VIIa and Xa (14). PAR1 is also shown to be activated by coagulation factor Xa (15). All of these receptors, with the exception of PAR3, respond not only to a selected protease but also to their respective tethered ligands and analogs (4, 6). PAR1 and PAR3 but not PAR4 have a high affinity for thrombin because only PAR1 and PAR3 contain N-terminal hirudin-like sequences. PAR3 does not signal on its own but serves as a coreceptor for PAR4 to facilitate PAR4 cleavage by thrombin (16). There is a marked species difference in regard to PAR profiles in human and rodent platelets: human platelets contain PAR1 and PAR4, whereas rodent platelets contain PAR3 and PAR4 (4).

PAR1 is widely distributed in many different cell types and tissues. PAR1 is found in platelets, vascular endothelial cells, smooth muscle cells, fibroblasts, monocytes, mast cells, T cells, neurons, astrocytes, glia, cardiac and skeletal myocytes, epithelial cells, keratinocytes, osteoblasts, *etc*. (6). Also, most mammalian cell lines examined (COS, HEK293, HeLa, RAT1, RAT2, HUVEC, hCASMC, CaCo2, HLF, *etc*.) contain a low level of PAR1. While endothelial cells contain a high density of PAR1 *in vivo*, normal vascular smooth muscle cells express relatively low levels of PAR1. PAR2 is also relatively widely distributed: endothelial cells, smooth muscle cells, leukocytes, mast cells, keratinocytes, fibroblasts, neurons, astrocytes, glia, osteoblasts, *etc*. Many cells contain more than one PAR (6), for example, PAR1 and PAR4 in human platelets, PAR1 and PAR2 in endothelial cells and cardiomyocytes (17), PAR1, PAR2 and, in case of saphenous vein, PAR4 (18) in vascular smooth muscle cells. Various tumor cells also express high levels of PAR1 and PAR1 activating proteases as well as other PARs.

Biochemistry and pharmacology of PAR1

Ligand binding and activating domains

Earlier studies employing antibodies directed to different parts of PAR1 and chimeras of human and *Xenopus*

Table I: Profiles of the family members of proteinase activated receptors.

Nomenclature	PAR1	PAR2	PAR3	PAR4
Agonist proteases[a]	Thrombin, trypsin, FXa, granzyme A	Trypsin, tryptase, FXa, TF/VIIa	Thrombin	Thrombin, trypsin, cathepsin G
Cleavage sequence[a]	LDPR/SFLLR	SKGR/SLIGK	LPIK/TFRGAP	PAPR/GYPGQV
Human tethered ligands[a] (analogues)	SFLLRN (TFLLRN, TFLL-RNPNDK)	SLIGKV	None known, no signaling	GYPGQV (AYPGQV)
Antagonists[b]	RWJ-56110, SCH-79797	None known	None known	*trans*-Cinnamoyl-YPGKF
Gene deletion in mice[a]	Partial embryonic lethality; normal platelet response to thrombin	Mild impairment of leukocyte migration	Inability of platelets to respond to low conc. of thrombin	Inability of platelets to aggregate in response to thrombin
Distribution in humans[c]	Widely distributed: platelet, monocyte, endothelial cell, smooth muscle cell, mast cell, T cell, fibroblast, astrocyte, neuron	Endothelial cell, leukocyte, smooth muscle cell, mast cell, astrocyte, myocyte, leukocyte, glia, neuron, epithelial cell, keratinocyte	Endothelium, lung, liver, leukocyte, GI tract, pancreas	Platelet, GI tract, lung, endothelium, leukocyte, pancreas

[a]Ref. 7, 9, 15. [b]Ref. 24, 25 and this article. [c]Ref. 6, 145, 150.

PAR1 identified the Glu260 region in the extracellular loop (ECL2) and to a lesser degree Phe87 in the N-terminus as important determinants of agonist specificity (9). An electrostatic interaction of ECL2 with the SFLLRN ligand peptide was suggested to occur between Glu260 and Arg46 in the tethered ligand domain. Insertion of eight residues of *Xenopus* ECL2 into human PAR1 produced a constitutively active receptor (19). Many early studies noted divergent effects of thrombin and PAR1 tethered ligand analog peptides (5). Although many of these differences were later found to be due to multiple thrombin receptors, there still remains a question of whether the tethered ligand and soluble PAR1 agonist peptide act differently. In one earlier study where the effects of PAR1 amino terminal mutations on both thrombin and TRAP activation were examined, similar attenuation of the responses was observed with the agonist peptide and thrombin (20). Most recently, a comprehensive study examined the effects of mutations on the functional activation of PAR1 induced by TRAP and thrombin as well as on the TRAP binding (21). Many point mutations (I88A, S89A, L96A, D256A, E347A), with the exception of D256N and E260A, and deletion of extracellular domains (δEC3[339-344], δNH3[68-93]) had a profound effect on the functional responses to the agonist peptide TRAP without significantly altering thrombin-induced responses or TRAP binding. These results demonstrated that there are profound differences in the activation of the PAR1 by the tethered ligand generated by thrombin cleavage *versus* the free TRAP peptide (SFLLRNP). D256A and C175S were only two single mutations that markedly affected the high-affinity TRAP [^{3}H]C721-40 binding. The C175S mutation also abolished the functional response to thrombin. Disulfide bonds linking extracellular domains of G-protein-coupled receptors have been shown to be important for maintaining the conformation of the receptor and to allow ligand access to the binding pocket (22). Thus, it was not surprising that a loss of binding and functional response was observed with this mutant. Taken together, these data indicate an involvement of D256 rather than E260 in TRAP binding in contrast to earlier results (20, 23). This study also points out the difference in the intrinsic efficacy of the tethered ligand and the soluble peptide.

Although it appears from this study that the PAR1 tethered ligand and a soluble ligand peptide bind different sites, several studies with PAR1 antagonists indicate effective and complete inhibition of thrombin-induced functional activity. Human coronary artery smooth muscle cells contain only one functioning thrombin receptor, PAR1, activation of which led to calcium mobilization and mitogenesis (24). In these cells, SCH-79797, an antagonist of tethered ligand analog binding, was able to completely inhibit thrombin- and a PAR1 agonist peptide-induced calcium transients and mitogenesis. Similarly, in rat aortic smooth muscle cells a peptidomimetic PAR1 antagonist also effectively inhibited thrombin-induced calcium transients and mitogenesis (25). Taken together, these data suggest that unlike mutations, antagonists affect binding and signaling of both the tethered ligand and soluble agonist peptide equally well, an assuring observation for industrial scientists involved in the development of PAR1 antagonists as therapeutic agents.

PAR1 activation, desensitization and resensitization

Cleavage of PAR1 by thrombin is irreversible and the peptide tethered ligand unmasked by cleavage remains tethered to the receptor. In the interaction of thrombin with PAR1, its hirudin-like domain functions to reduce the kinetic barriers to formation of the docked thrombin-PAR1

complex (26). The exosite 1-bound hirudin motif facilitates the productive interaction of the PAR1-LDPR/SFL (residues 38-44) sequence with the active site of thrombin. This locking process is the most energetically unfavorable step of the overall reaction. The subsequent irreversible steps of peptide bond cleavage are rapid.

Like other G-protein-coupled receptors, activated PAR1 is rapidly uncoupled from signaling and internalized by phosphorylation-dependent mechanisms (4). Internalized receptor then moves to lysosomes for degradation. In fibroblasts and endothelial cells, thrombin responsiveness is maintained by delivery of new PAR1 to the cell surface from an intracellular pool of PAR1 (27, 28). Like fibroblasts and endothelial cells, human coronary artery smooth muscle cells contain an intracellular pool of PAR1, which rapidly replace surface PAR1 lost due to internalization. By contrast, human megakaryoblastic cell lines have no intracellular reserve of PAR1 and recovery of PAR1 signaling requires new protein synthesis (29). This may reflect no need for resensitization of PAR1 in the platelet which is derived from megakaryocytes since platelets are activated once and are incorporated into a clot.

Since even low concentrations of thrombin would be expected to eventually cleave all the available receptors on the cell surface, the question arises of how might a cell detect differences in thrombin concentrations. The rapid shut-off of activated PAR1 provides an answer to how PAR1 mediates graded responses to varying concentrations of thrombin (30). Each cleaved receptor is active for a finite interval to produce certain amounts of second messengers. Because the second messenger is itself cleared, the level of second messenger achieved is proportional to the rate at which receptors are cleaved and thus to thrombin concentration. This rapid shut-off mechanism as well as the apparent low affinity of the tethered ligand for PAR1 makes it feasible to develop effective PAR1 antagonists. Thus, in order to attenuate cellular responses, an antagonist need only delay PAR1 activation. Indeed, PAR antagonists that effectively compete with tethered ligand for receptor have been developed (24, 25, 31).

Transactivation of PAR1 and PAR2

Although transactivation of PAR1 was first noted in an early study employing chimeras, this has no pharmacological consequence due to its very low efficiency relative to intramolecular thethered liganding mechanism (32). The ability of a soluble tethered ligand peptide (SFLLRN) to activate PAR1 and PAR2 (33, 34) raises the possibility of activation of PAR2 by thrombin-cleaved PAR1. To see whether this could occur, thrombin responses were examined in COS-7 cells coexpressing PAR2 with a PAR1 variant (L258P) that can be cleaved but not activated by thrombin because of the mutation in ECL2. When the receptors were coexpressed, robust thrombin signaling was observed (35). To determine whether transactivation could occur on endothelial cells with naturally occurring forms of two receptors, HUVEC were preincubated with the PAR1 antagonist, BMS-200261, at a concentration that completely blocked thrombin signaling on cells expressing only PAR1. Even at high concentrations, this antagonist blocked at most 75% of the response to thrombin. Desensitization of PAR2 blocked the remaining 25% of the thrombin response (35). These results suggested that PAR2 can be activated by cleaved PAR1 on endothelial cells and allows PAR1 to contribute to the thrombin response at least when a selective PAR1 antagonist is present.

Transactivation of PAR2 by PAR1 has several implications. First, it suggests that PAR1 and PAR2 on the endothelial cell surface are located sufficiently close enough to each other to allow the cleaved N-terminus of PAR1 access to PAR2. Secondly, it has implications for the development of PAR1 antagonists. On human platelets, the efficacy of a PAR1 antagonist is limited by the presence of PAR4. The transactivation studies predict that on cells expressing both PAR1 and PAR2, the efficacy of PAR1 tethered ligand antagonists will be limited by the presence of PAR2 even if other thrombin receptors are not present. Human coronary artery smooth muscle cells contain both PAR1 and PAR2 that are functionally coupled to calcium mobilization. In these cells, SCH-79797 almost completely blocked both thrombin- and a selective PAR1 agonist peptide-induced calcium transients (24). This suggests minimal to no significant transactivation of PAR2 by cleaved PAR1 in human coronary smooth muscle cells in contrast to HUVEC. The cell-specific transactivation may reflect a different density of surface PAR1 and PAR2 in these two types of cells and suggests a possibility that the efficacy of a PAR1 antagonist may vary among different cell types.

Signaling

Although the exact intracellular signaling cascade is cell-type specific, a general scheme can be drawn. Most of PAR1 signaling is mediated through heterotrimeric G proteins. PAR1 is promiscuous, coupling to multiple G proteins in the same cell (*e.g.*, human platelets) including G_i, G_q and $G_{12/13}$ (36). G_q-dependent signaling activates phospholipase C, which leads to mitogen-activated protein kinase (MAPK) phosphorylation and receptor tyrosine kinase transactivation, both necessary events in thrombin-mediated proliferation. Gbetagamma interactions activate phosphoinositide-3-kinase, which promotes Ca^{2+} release that is required for smooth muscle cell growth in response to thrombin (37). G_{13}-dependent signaling activates Rho activation that causes cytoskeletal changes affecting membrane ruffling and vascular cell migration (38, 39). G_i-dependent signaling inhibits adenylate cyclase leading to a decrease in cAMP levels in vascular smooth muscle cells (40).

A number of recent studies have indicated crosstalk between PAR1 and receptor tyrosine kinases or integrins

(6, 41, 42). Thrombin was found to activate the EGF receptor (43). Initially it was believed that this cross talk was not mediated by EGF. More recent studies have elucidated a novel mechanism of EGF receptor activation. PAR1 activation by thrombin induces cell surface metalloproteinase activity that cleaves and releases the active ectodomain of heparin-binding EGF-like growth factor (HB-EGF), which then activates the EGF receptor (44, 45). In Rat 1a fibroblasts, PAR1 activation significantly stimulated Shc and FAK tyrosine phosphorylation and Shc-Grb2 and FAK-Grb2 complex formation (46). Tyrosine phosphorylation of Shc is inhibited by pertussis toxin treatment and is insensitive to cytochalasin D. The rapid stimulation of Erk1/2 correlates with tyrosine phosphorylation of Shc but not FAK. Further, PAR1 mediated Erk1/2 activation is endocytosis-dependent because the activation was inhibited by inhibitors of clathrin-mediated endocytosis. Thrombin also appears to activate the PDGF receptor via PAR1 by inducing PDGF synthesis and release, which stimulates PDGF receptor in an autocrine or paracrine fashion (47). Recent studies revealed interaction of PAR1 with integrins. β_3 Integrins are upregulated after vascular injury and modulate thrombospondin- and thrombin-induced proliferation of cultured smooth muscle cells (42). This integrin signaling is necessary for maximal thrombin-mediated proliferative events in smooth muscle cells. In this respect it is interesting to note that the successful antithrombotic gpIIb/IIIa antagonizing agents abciximab and eptifibatide have been found to block β_3 integrins (48).

Nonproteolytic PAR1 activation and non-PARs

Although cellular effects of thrombin require its proteolytic activity (for cleavage of PAR1) in most cells (4), exceptions were earlier noted for certain types of cells. Proteolytically inactive thrombin could elicit chemotaxis and mitogenesis in J774 macrophage-like cells (49) and, together with either gamma-thrombin or the PKC activator phorbol myristate acetate, stimulated mitogenesis in NIL hamster fibroblasts (50). Initiation of fibroblast proliferation by thrombin appeared to require two separate types of signals: one generated by proteolytic cleavage and the other by high-affinity cell surface binding. These actions of catalytically inactive thrombin were attributed to two functional thrombin domains lying outside of the catalytic site (51). The functional sequences within the active thrombin domains comprise residues 367-380 of the thrombin β-chain (YPPWNKNFTENDLL: TDP-1) and residues 508-530 of human prothrombin (AGYKPDEGKRGDACEGDSGGPFV: TDP-2). These peptides were shown to compete with ^{125}I-labeled thrombin for cell surface binding site (51, 52). TP-508 (TDP-2) binds to high-affinity thrombin binding sites and enhances the ability of thrombin to stimulate DNA synthesis or stimulates DNA synthesis in cells treated with 25 ng/ml phorbol myristate acetate or γ-thrombin (50, 52). TP-508 enhances wound healing and neovascularization in normal rats (53). A recent study has demonstrated that TP-508 activated a cellular response (expression of annexin V) separate from that activated through PAR1 and supported the hypothesis that TP-508 acts through a separate nonproteolytically activated thrombin receptor (54). TDP-1 and a longer peptide F-14 induced proliferation of HUVEC (55). Mitogenic effects of the PAR1 tethered ligand peptide $TRAP_{1-14}$, but not F-14 were inhibited by two antibodies directed against TRAP or the cleavage site, indicating the different sites of binding for these two peptides. Also, the mitogenic effects of $TRAP_{1-14}$ and F-14 were additive (55). Although these results are consistent with an action of F-14 on a non-PAR, a more recent study with a PAR1 oligonucleotide demonstrated that mitogenic effects of both $TRAP_{1-14}$ and F-14 are mediated via PAR1 (56). Interestingly, even though F-14 binds to PAR1, F-14 failed to induce the responses classically associated with activation of PAR1 such as intracellular Ca^{2+} release, cell monolayer permeability increase or PGI_2 release. In hamster CCL-39 fibroblasts neither TDP-1 nor TDP-2 alone had any effect on thymidine incorporation (57). However, TDP-1 considerably augmented $TRAP_{1-5}$ amide-mediated thymidine incorporation in this cell. Proteolytically inactive thrombin also acted synergistically with $TRAP_{1-5}$ amide to stimulate CCL-39 cell thymidine incorporation. These data taken together suggest that thrombin can cause its cellular effects, such as thymidine incorporation, not only via the proteolytic activation of PAR1 but also via the concurrent and synergistic interaction of its TDP-1 or TDP-2 peptide domain with a separate cell surface docking site. In human coronary artery smooth muscle cells this type of dual mechanism of action of thrombin does not appear to operate since α-thrombin and a PAR1 selective agonist peptide TFLLRNPNDK (33) cause a similar degree of stimulation of mitogenesis (24). Further, the mitogenic effect of thrombin and the peptide could be completely blocked by SCH-79797, a selective PAR1 antagonist. By contrast, in bovine coronary artery smooth muscle cells thrombin but not a PAR1 agonist peptide stimulated mitogenesis (58), raising the possibility of a dual mechanism of thrombin action.

Thrombin cleaves its PAR1, thereby releasing a 41-amino acid peptide as well as generating a new amino terminus that acts as a tethered ligand for the receptors. The 41-amino acid cleaved peptide TR_{1-41} was shown to induce platelet aggregation and was more efficacious than the tethered ligand peptide $TRAP_{1-14}$ (59). TR_{1-41} appears to bind to a non-PAR thrombin receptor since this peptide has no proteolytic action and is synergic with $TRAP_{1-14}$ in activating platelets. While the effective concentrations of this peptide are in the micromolar range (59), only a subnanomolar quantity of this peptide is cleaved and released from platelet PAR1 by a high concentration (10 nM) of thrombin (60). The big difference between the effective concentrations and the available concentration of this peptide makes the physiological significance of platelet activation by TR_{1-41} unclear.

The platelet gpIb-IX-V complex represents still another non-PAR thrombin receptor (see the next section).

PARs and platelet activation

Human platelets express PAR1 and PAR4, with activation of either sufficient to trigger platelet secretion and aggregation (2, 11, 61). Studies with selective neutralizing antibodies have indicated that PAR1 mediates activation of human platelets at low thrombin concentrations and that, in the absence of PAR1 function, PAR4 can mediate platelet activation but only at high thrombin concentrations (36). Unlike PAR1, PAR4-mediated platelet aggregation requires the presence of an ADP receptor. Platelets contain two G-protein-coupled ADP receptors: $P2Y_1$, a G_q-coupled receptor involved in calcium mobilization and shape change, and $P2Y_{12}$, a recently cloned G_i-coupled receptor, which lowers cAMP levels to allow platelet activation (62). PAR1 appears to be the main human thrombin receptor under the normal situation with PAR4 serving as a backup system. PAR4 may also be activated by proteases other than thrombin such as cathepsin G, a granzyme released by activated neutrophils (63). Human platelets also respond to γ-thrombin via PAR4. PAR4 may be responsive to *in vivo* generated γ-thrombin (64). The equal intensity of platelet responses as well as synergy between gpIb and PAR1 and/or PAR4 were observed *in vitro* with low levels of α-thrombin acting on PAR1 and gpIb (64). The ability of α-thrombin to bind and activate gpIb and simultaneously activate PAR1 and/or PAR4 may explain the synergistic potential of these three thrombin receptors. PAR4 is activated and shut off more slowly than PAR1 and the magnitude of calcium signaling in response to thrombin in human platelets appears to represent the sum contribution of both receptors (65, 66). A surprising finding was recently reported that platelet activation with the PAR1 tethered ligand SFLLRN resulted in PAR1 cleavage and release of TR_{1-41} (67). Both PAR1 cleavage and platelet activation induced by SFLLRN was markedly inhibited by the serine protease inhibitor pefabloc SC and soybean trypsin inhibitor. Thus, trypsin-like platelet protease propagates SFLLRN-dependent PAR1 cleavage and platelet activation (67). It is not known whether this mechanism plays any role in *in vivo* situations or operates in cell types other than platelets.

In contrast to human platelets, mouse platelets express PAR3 and PAR4 (61, 68). Indeed, earlier studies showed that PAR1 activating peptides activated human but not murine platelets (69-71). Knockout of mouse PAR1 had no effect on thrombin signaling in mouse platelets but abolished thrombin signaling in fibroblasts (71). In mouse platelets, knockout of mouse PAR3 revealed PAR3 to be necessary for activation at low concentrations of thrombin. Remaining thrombin signaling in PAR3-deficient mouse platelets was attributed to murine PAR4 (68). Whereas expression of mPAR3 in COS cell did not, by itself, confer thrombin signaling, coexpression of mPAR3 with mPAR4 reliably enhanced both mPAR4 cleavage and signaling at low concentrations of thrombin compared with mPAR4 alone (16). The N-terminal exodomain of mPAR3 and, more specifically, its thrombin-interacting sequence were necessary. Thus, it appears that mPAR3 does not signal on its own but functions as a coreceptor for cleavage and activation of mPAR4 at low thrombin concentrations. This model predicts that thrombin signaling in mouse platelets is dependent on PAR4. Indeed, this case has been shown by PAR4 knockout which renders mouse platelets unresponsive to thrombin (72).

The platelet gpIb-IX-V is a major complex on the platelet surface and plays a critical role in thrombus formation under conditions of high shear (73). Absence of gpIb-IX-V results in a severe bleeding disorder known as Bernard Soulier syndrome characterized by giant platelets and impaired von Willebrand factor binding (74). Thrombin binds to the platelet surface glycoprotein $gpIb_\alpha$. Recently, a role for gpIb in platelet procoagulant activity was proposed based on the observations that inhibition of thrombin binding to gpIb inhibited annexin V binding to platelets (75). In earlier work, evaluation of platelets from gpV null mice indicated that absence of the thrombin substrate gpV from the complex rendered the platelets more responsive to thrombin and gpV null mice were found to have a shorter bleeding time (76). The latest study has shown that the absence of gpV (induced either by knockout or cleavage by a nonactivating concentration of thrombin) results in the ability of platelets to signal through $gpIb_\alpha$ upon thrombin or DIP-thrombin (a proteolytically inactive thrombin) binding (77). DIP-thrombin at a concentration of 400 nM can induce platelet aggregation in gpV null platelets *in vitro* and *in vivo* and inhibitors of thrombin binding to $gpIb_\alpha$ ablated the aggregation response (64, 77). Apparently, conflicting observations were observed for a role of gpIb in two different studies with gpV null mice (77, 78). This apparent conflict was partially resolved when agents such as PGE_1, which activates adenylate cyclase, completely inhibited the aggregation induced by proteolytically inactive thrombin but not by native thrombin, suggesting that repression of adenylate cyclase is critical for platelet activation through the gpIb-IX complex (77). Kahn's but not Ramakrishnan's laboratory included PGE_1 and EDTA, which would inhibit platelet aggregation via activation of gpIb-IX but not PARs. On the other hand, complete inhibition of DIP-thrombin-mediated platelet aggregation by a $P2Y_{12}$ inhibitor supports a role for secreted ADP in platelet activation via the thrombin-$GPIb_\alpha$ interaction, and specifically a role for $P2Y_{12}$. Systemic administration of proteolytically inactive thrombin to gpV null mice results in dramatic induction of thrombosis that does not occur in wild-type mice (77). These results show that inactive forms of thrombin bind to gpV-deficient platelets *in vivo* and activate platelet aggregation, leading to thrombosis. PAR1 antagonists (RWJ-56110 and SCH-203099) have minimal effect on platelet aggregation induced by DIP-thrombin in gpV null mice, suggesting their lack of effects on DIP-thrombin-induced signaling via gpIb-IX (64, 77). These data, together with the observation (64) of a marked synergistic action between PAR1 and gp1b, indicate an important role of gp1b in platelet activation by thrombin.

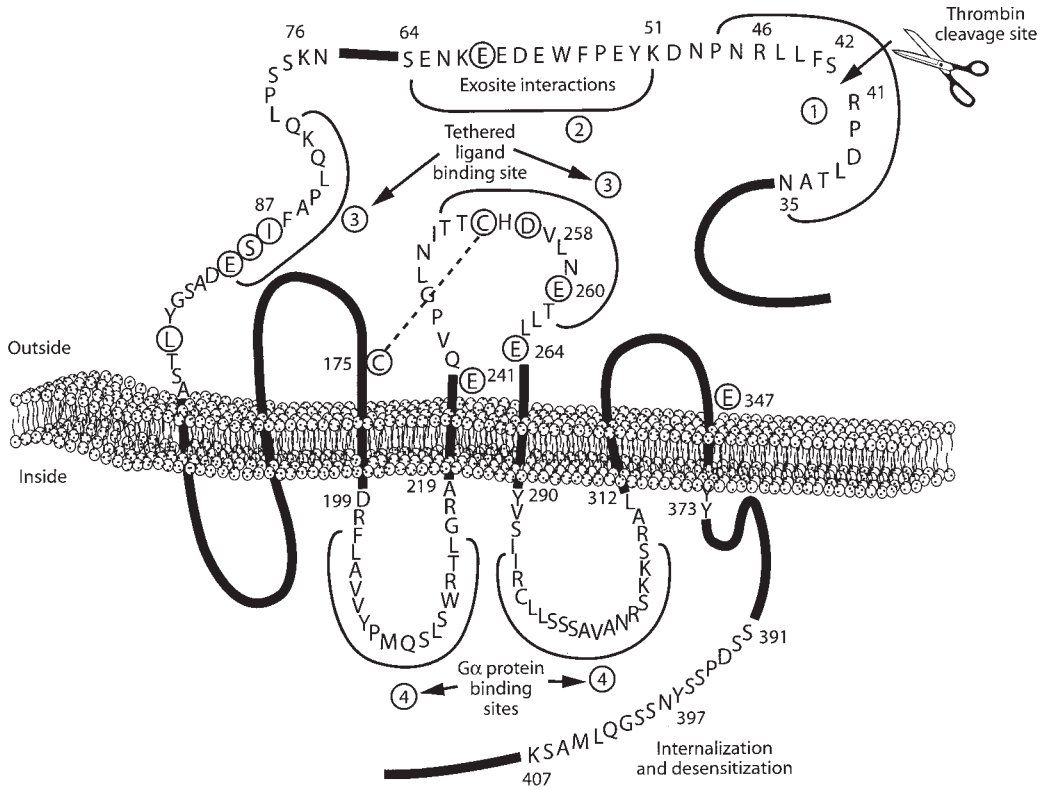

Fig. 1. Schematic drawing of human PAR1 highlighting potential sites of PAR1 inhibition. *Site 1:* Blocking of this site would prevent cleavage between R41 and S42 residues by thrombin. However, SPAN12, a monoclonal antibody directed to this site only partially inhibits thrombin-induced platelet aggregation without inhibiting platelet aggregation induced by a soluble PAR1 agonist peptide. *Site 2:* Blocking of this site may block high affinity binding of thrombin to PAR1. The monoclonal antibody to this site only weakly inhibits platelet aggregation. However, a polyclonal antibody effectively inhibited thrombin-induced platelet aggregation and exhibited antithrombotic effects in African green monkeys (80). *Site 3:* These tethered ligand-binding sites have proven to be the best target for developing PAR1 antagonists, yielding several classes of nonpetide antagonists (95, 97). *Site 4:* These sites denote intracellular loops 2 and 3 that interact with Gα protein. The intracellular loop 2 is involved in the interaction with $G\alpha_q$ while loop 3 interacts with $G_{o/i}$ subunits. Introduction of minigene vectors that encode the C-terminal peptides ($G\alpha_i$, $G\alpha_q$, $G\alpha_{12}$ and $G\alpha_{13}$) into human microvascular endothelial cell line selectively antagonize G protein signal transduction events (81). The C-terminal peptides serve as competitive inhibitors presumably blocking the G-protein-coupled receptor that normally binds the G protein. Circles show the mutation sites in which replacement by alanine significantly altered the ability of TRAP or both thrombin and TRAP to activate the receptor. The arrow indicates the thrombin cleavage site between R41 and S42. The seven-amino acid residue distal to the thrombin cleavage represents the first seven-amino acid residues of the tethered ligand (SFLLRNP).

The presence of these multiple thrombin receptors and synergy between them in human platelets raises an important question regarding the development of antithrombotic drugs: might inhibition of PAR1 be sufficient to prevent thrombosis? Early *in vivo* studies showed that inhibition of PAR1 with a PAR1 neutralizing antiboy or an antagonist had an antithrombotic effect in nonhuman primates (79, 80). These results indicate a predominant role of PAR1 over various non-PAR thrombin receptors in thrombogenesis.

Types of PAR1 inhibitors

PAR1 signaling may be inhibited in many different ways. Figure 1 depicts various sites of action for PAR1 antagonists. Not shown in the diagram is the site of thrombin inhibitors that block PAR1 cleavage and activation by thrombin. Although direct thrombin inhibitors have antithrombotic effects, their inhibitory action on hemostasis and hence bleeding side effects hampered development of this class of inhibitors as antithrombotic agents. The site 1 and 2 binding agents would prevent thrombin from binding to the cleavage site and the hirudin-like domain of PAR1, respectively. WEDE15, a monoclonal antibody to the hirudin-like domain, partially inhibits thrombin binding although hirudin, which binds thrombin's exosite 1 with a higher affinity, completely inhibits thrombin activation of PAR1. A combination of SPAN12, a monoclonal antibody to the N-terminal amino acid residues 35-46 of PAR1, and WEDE15 completely inhibits thrombin binding and cleavage of PAR1 (28). The site 3 binding inhibitors represent antagonists of PAR1 tethered

ligand and so far appear to be most effective in blocking PAR1 activation by thrombin and soluble agonist peptides (6, 24, 25). The site 4 binding inhibitors would selectively block PAR1 interaction with G protein. (81). Still another type of inhibitors are inhibitors of specific kinases involved in the PAR1-mediated signaling pathway (*e.g.*, Src, NF-κB). These inhibitors pose specificity problems since kinases are involved in other G-protein-coupled receptor-mediated signaling as well.

Development of PAR1 and PAR2 binding assays

Initially it was thought that developing PAR1 antagonists might not be possible because the tethered ligand would have a great entropic advantage over exogenous added antagonists (82). In view of a great medical need for an effective antithrombotic agent, its large market potential and the known potent platelet activating action of thrombin, the majority of companies have focused on developing thrombin receptor antagonists as antithrombotic agents. In the course of setting up *in vivo* assays they became aware of species differences in platelet responses to PAR1 activating tethered ligand or its analogs (SFLLRN or haTRAP) (69, unpublished data). These PAR1 agonist peptides could activate only human, monkey and, to a lesser extent, guinea pig platelets. Hence, this species difference necessitated the use of human platelets for *in vitro* assays and of nonhuman primates for *in vivo* assays. Although the usual way of screening compounds for any receptor antagonist is the use of a receptor ligand binding assay, the lack of a high-affinity tethered ligand prevented development of a thrombin (PAR1) receptor binding assay. Initially, various functional assays including arachidonate release from HUVEC and human platelet GTPase assays were used to screen thrombin receptor antagonists without much success, probably due to the lack of sensitivity and large interexperimental variations. Active natural products were discovered using an intracellular calcium mobilization assay with HeLa cells (83). With the availability of haTRAP developed by Feng *et al*. (84), we were able to successfully develop a thrombin receptor [^{3}H]haTRAP binding assay (85). While the signal to noise ratio of the [^{3}H]haTRAP binding assay was 6-10, that of [I^{125}]haTRAP was less than 2 due to high nonspecific binding. At that time we did not know that tethered ligand peptide and haTRAP have affinity for PAR2 (86). Fortunately for us, human platelets did not have PAR2 and hence the binding assay was valid and could be used to identify PAR1 antagonists. However, we soon found that this binding assay did not work if we used membranes from cell types other than human platelets. This puzzle was solved later when several reports came out demonstrating the presence of both PAR1 and PAR2 in various cell types (5). The [^{3}H]haTRAP binding assay is more sensitive and robust than functional assays. This assay allows (i) the use of DMSO concentrations higher that those used in any cell-based assay which permits better solubilization of poorly soluble compounds, (ii) a longer incubation so as not to miss slow binding antagonists and (iii) the use of samples containing multiple compounds, which speeds up the screening process. The haTRAP binding assay can be performed using membranes prepared from Sf9 cells expressing human PAR1 but it is less sensitive to antagonists than the standard binding assay using human platelet membranes (Foster and Leyla, unpublished observation). Recently, other PAR1 tethered ligand binding assays have been reported which utilized different high-affinity tethered ligand analogues (21, 25). Unlike PAR1 transfected Sf9 cells, PAR1 on the transfected COS7 cells exhibited binding affinities for PAR1 agonists and an antagonist BMS-200261, which are similar to those of human platelet PAR1 (21). The signal to noise ratios of these assays is either comparable to or lower than that of our haTRAP binding assay.

Accumulating evidence indicates an important role of PAR2 besides PAR1 and the potential therapeutic utility of PAR2 antagonists. Recently, a PAR2 tethered ligand binding assay was developed using a tethered ligand derivative which is somewhat more potent than the tethered ligand and an oil-aqueous partition method (87). Because of its labor-intensive steps – centrifugation and transfer of a separated phase to a counting vial – it will be difficult to adapt this assay for a high-throughput screening assay. A high-affinity PAR2 ligand (K_d of 1-10 nM) may be necessary for development of a high-throughput multiwell plate-based PAR2 binding assay.

Chemistry of PAR1 antagonists

The currently available thrombin receptor antagonists can be classified into three categories: peptide antagonists, peptidomimetic antagonists and nonpeptide thrombin receptor antagonists.

Peptide antagonists

The development of peptide antagonists for the thrombin receptor evolved around the SAR studies of the tethered ligand (88). It was originally found that the 14-amino acid containing peptide amide, SFLLRNPNDKYEPF-NH_2, which mimics the sequence of the N-terminal portion of the tethered ligand, was a full agonist (84). Novel thrombin receptor analogs, with potent agonist effects, capable of activating the receptor in the absence of thrombin and eliciting functional responses such as platelet aggregation, GTPase turnover and intracellular calcium mobilization, were subsequently developed. 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 (89). Optimization of the agonist property of the pentapeptide was achieved by substituting phenylalanine at position 2 with nonproteogenic amino

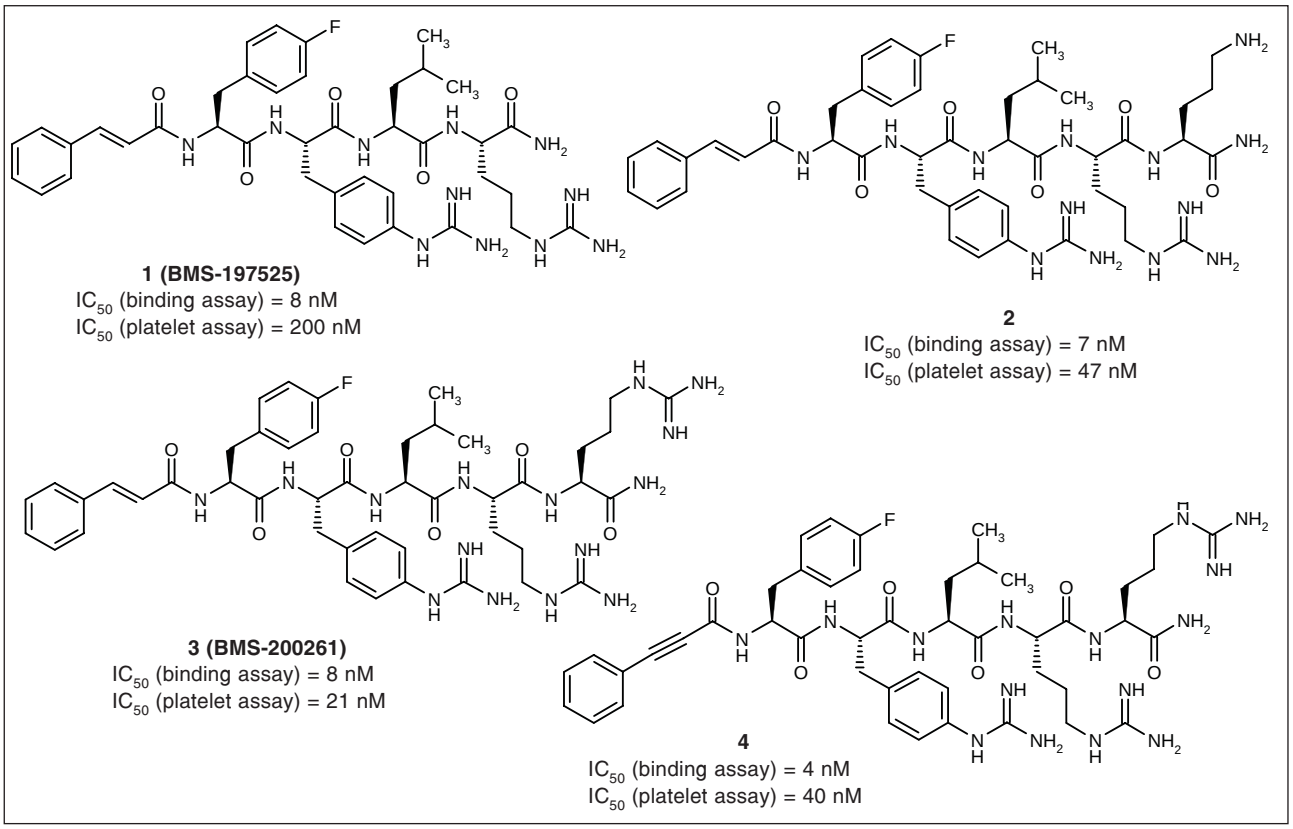

Fig. 2A. PAR1 peptide antagonists.

acids. SF(f)LLR-NH_2, in which the phenylalanine residue at position 2 is substituted by a *p*-fluorophenylalanine (90, 91), showed 4- to 5-fold higher activity in the phosphoinositide turnover assay in human epithelial cells. Substitution at position 3 of SF(f)LLR-NH_2 by *p*-guanidophenylalanine produced SF(f)F(Gn)LR-NH_2 which is the most potent pentapeptide known and displays full agonist activity. Further exploring the minimum structural requirement for the agonist activity, the des-Arg tetrapeptide analog of SF(f)F(Gn)LR-NH_2 was found to be an agonist with an EC_{50} of 0.28 μM, which is more potent than the natural pentapeptide. SF(f)F(Gn)LR-NH_2 has served as the basic structural motif for the development of peptide antagonists for the thrombin receptor (31, 92).

Antagonists were designed from the structure of the optimized pentapeptide SF(f)F(Gn)LR-NH_2 incorporating an early observation that certain acylations of the N-terminus would confer antagonistic properties (93). The most potent antagonists were generated by replacement of the serine residue with a *trans*-cinnamoyl group. Compound **1** showed an IC_{50} of 8 nM in the radioligand binding assay against the thrombin receptor. Extension of the C-terminus with residues containing basic side chain, ornithine and arginine, gave analogs **2** and **3** with comparable binding affinity, but improved potency in the agonist-induced platelet aggregation inhibition assay. Peptide **4**, replacing the *trans*-cinnamoyl group with a 3-phenyl-2-propynonyl group, has been the tightest binding thrombin receptor antagonist reported (IC_{50} = 4 nM) (31). (Fig. 2A).

Peptidomimetic antagonists

Based on the SFLLRN motif of the tethered ligand, researchers at R.W. Johnson and Cor Therapeutics identified peptidomimetic thrombin receptor antagonists (25, 94). It was discovered that the agonist peptide requires 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. Based on distance parameters taken from models of SFLLRN and low energy conformations, a three-point model relating the distance among the amino terminus, benzene ring of phenylalanine and the central carbon of arginine guanidine group was constructed. A 6-amino indole template was selected as a rigid molecular scaffold for spatially displaying the three key functional groups. This approach initially led to the identification of compound **5** which exhibited IC_{50} values of 0.7 and 0.3 μM, respectively, in a radioligand binding assay and a platelet aggregation inhibition assay using SFLLRN-NH_2 as the agonist (94). Further optimization led to compound **6** with improved potency in both thrombin and TRAP-induced platelet aggregation inhibition assays. However, these compounds manifested unexpected hypotensive effects in a

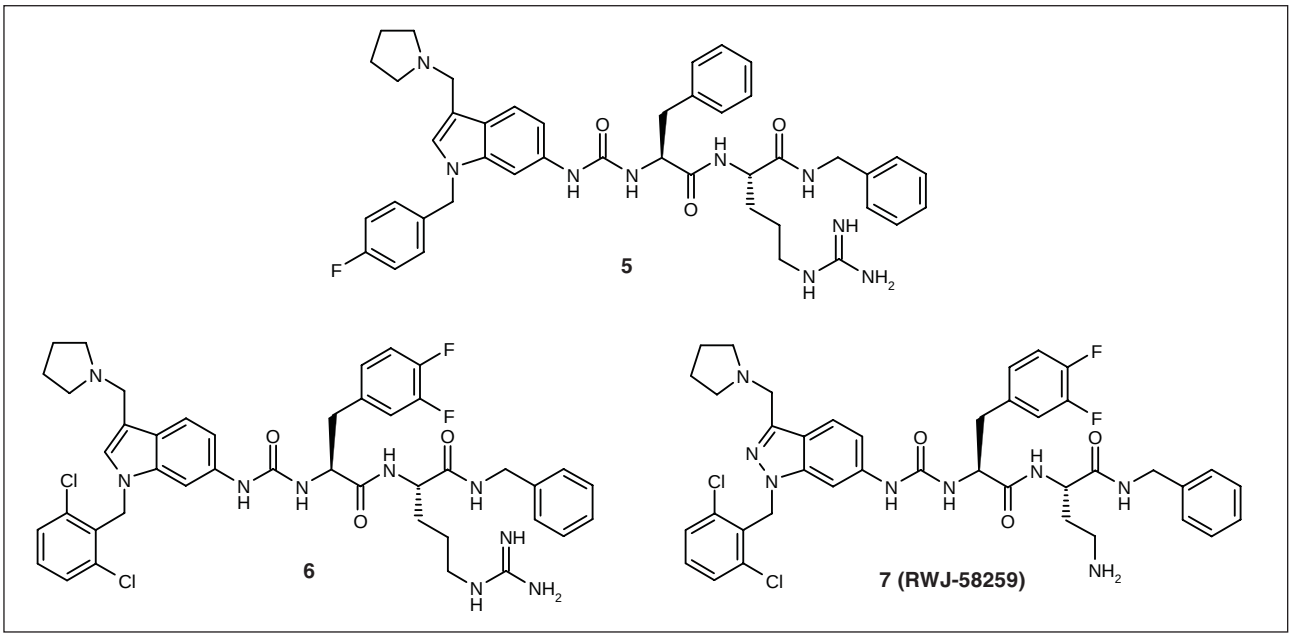

Fig. 2B. PAR1 peptide mimetic thrombin receptor antagonists.

guinea pig efficacy model. This problem was circumvented by replacing the indole moiety with an indazole group to generate compound **7** (RWJ-58259) (94). In the platelet aggregation inhibition assay, RWJ-58259 gave IC_{50} values of 0.57 and 0.18 μM against thrombin and peptide agonist, respectively. Although this compound was inactive in two standard *ex vivo* models in guinea pigs, which the authors attribute to the activation of PAR4 receptors, it blocked thrombin-induced calcium mobilization and smooth muscle cell proliferation in rat (which contains only PAR1). More importantly, RWJ-58259 showed significant reduction of neointima thickness in a rat restenosis model after perivascular administration, indicating that a thrombin receptor antagonist could have therapeutic utility in the treatment of vascular disorders such as restenosis and atherosclerosis (Fig. 2B).

Nonpeptide antagonists

The pyrroloquinazoline analogs represented by structures **8-10** were reported by Schering-Plough (Fig. 2C) (95). These are potent compounds that showed very specific SAR. A *p*-isopropylbenzyl group at N_7, a free amino group at C_2 and a substituted amino group at C_3 were required for reasonable affinity. Compounds **8** and **9** showed strong affinity in the radioligand binding assay and good potency in the TRAP-induced *ex vivo* platelet aggregation inhibition assay. Additionally, these compounds inhibited thrombin- and peptide agonist-induced elevation of Ca^{2+} transients in smooth muscle cells. Further characterization of their mode of action established that these compounds inhibited both TRAP, in a competitive manner, and thrombin-induced [^{3}H]thymidine incorporation in the mitogenesis assay (24). These studies conclusively prove that the biochemical and pharmacological effects of these compounds are mediated via inhibition of the PAR1 receptor.

FR-171113 (**11**) is another nonpeptide thrombin receptor antagonist. It inhibited thrombin- and peptide agonist-induced human platelet aggregation with IC_{50} values of 0.29 and 0.15 μM respectively (96). The development of this compound as a clinical candidate for antithrombotic therapy has reportedly been halted. Eryloside F (**12**), a steroidal disaccharide has been reported to be a thrombin receptor antagonist manifesting inhibition of agonist-induced platelet aggregation (81). However, the specificity and thrombin receptor affinity of this compound have not been reported.

Benzimidazole derivatives **13** and **14** have been reported to be high-affinity thrombin receptor antagonists with potent haTRAP- and thrombin-induced platelet aggregation inhibition (97, 98). In the binding assay, these compounds showed IC_{50} values of 65 nM and 33 nM respectively. Compound **13** inhibited haTRAP- and thrombin-induced platelet aggregation with IC_{50} values of 265 and 600 nM, respectively. Researchers at Merck have reported phenylisoxazole derivatives (**15**) as low-molecular-weight thrombin receptor antagonists (99). These compounds are reported to have IC_{50}s in the submicromolar range against TRAP-induced platelet aggregation. Thrombin receptor antagonists based on the natural product himbacine have been reported by Schering-Plough (100). For example, the tricyclic derivative **16** showed an IC_{50} of 15 nM against thrombin receptor in the binding assay.

Pharmacology of PAR1 antagonists

Table II lists many of the known PAR1 antagonists and their inhibitory activities in both *in vitro* binding studies and functional assays.

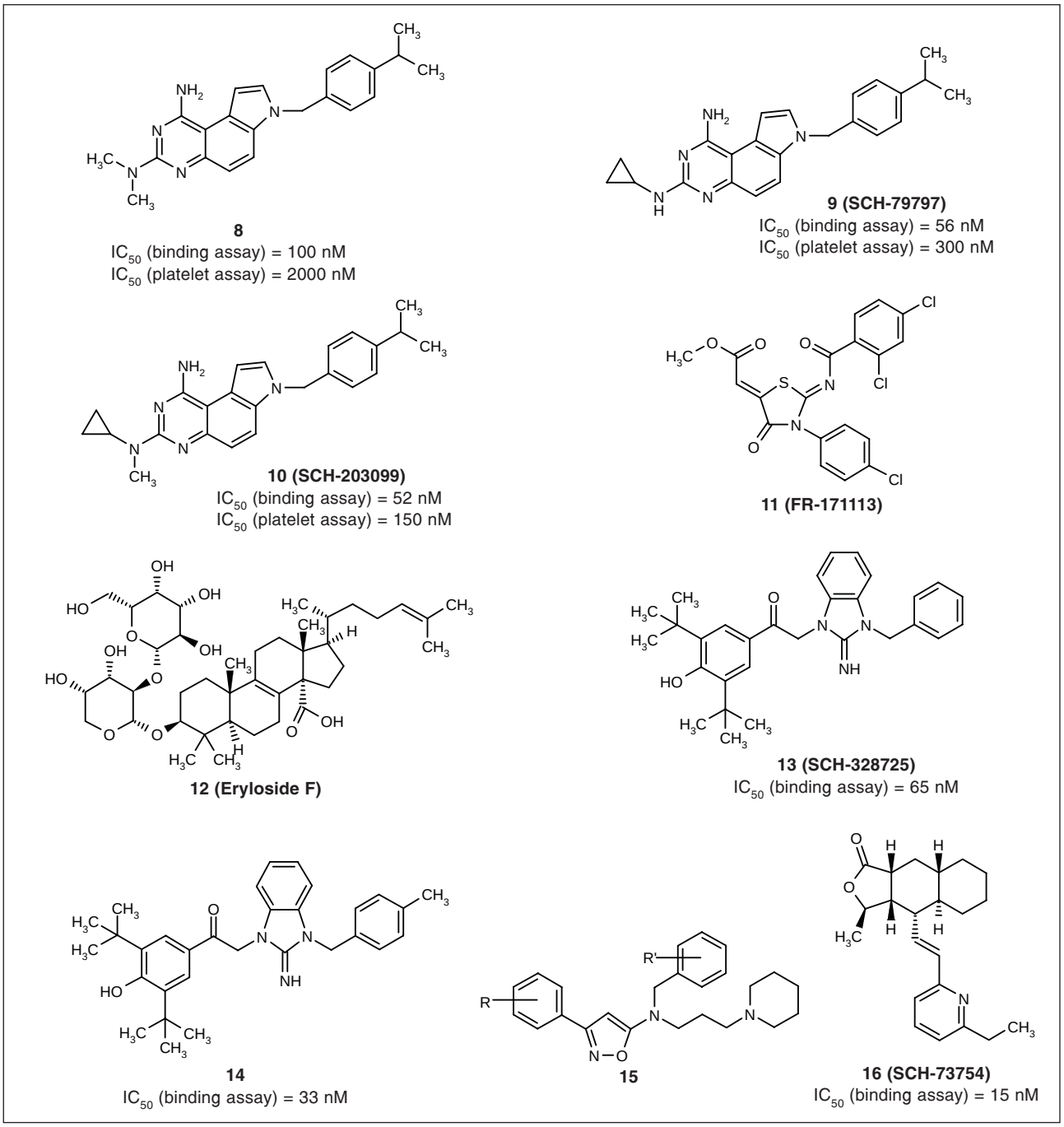

Fig. 2C. PAR1 nonpeptide thrombin receptor antagonists.

Peptide and peptidomimetic antagonists

Most peptide and peptidomimetic PAR1 antagonists have many drawbacks such as weak potency, inability to block thrombin activation of platelets, mixed agonist/ antagonist activity and/ or lack of PAR selectivity (31, 100-103). YFLLRNP, AFLARAA and 3-mercapto-propionyl-Phe-Cha-Arg-Lys-Pro-Asn-Asp-Lys amide (C186-65) are weak to moderate PAR1 antagonists with partial agonist activity. AFLARAA showed weak agonist activity (EC_{50}, 700-1000 μM), moderate antagonist activity (IC_{50}, about 24 μM against an agonist peptide SFLLRNA; IC_{50} <16 μM against thrombin) and some degree of inhibitory selectivity for thrombin- *versus* ADP- or collagen-induced platelet aggregation (104). The ability of this peptide to block agonist-induced platelet aggregation increased with the length of preincubation. This peptide also inhibited thrombin- and thrombin receptor activating peptide-

Table II: Inhibition of PAR1 by various PAR1 antagonists.

PAR1 antagonist	Assay type	Effect IC_{50}, K_i[a] or K_b[b], μM	Ref.
Peptide antagonists			
FLLRN	pa	< 200	107
	pb	> 200	UD
LVR(D-)CGKHSR	ra	180 (Tb or TRAP14)	146
MSRPACPNDKYE	pa	100-180 (Tb)?	106
	pb	> 200	UD
YFLLRNP	pa	100 (SN)	149
	pb	29[a]	UD
AFLARAA	pa	16-33 (SA) < 16 (Tb)	104
Peptidomimetic antagonists			
3-Mercapto-propionyl FChaChaRKPNDK-NH_2	pa	0.7-6.4 (Tb)	87 101
	pb	8.9[a]	86
BMS-197525	pa	0.2 (SP)	31
	pb	0.01-0.03	
	smc	0.27[b]	UD
BMS-200261	pa	0.02 (SN) 1.6 (Tb)	31 87
S(Npys)-βMp-(p-F)F-NHCH$(C_6H_5)_2$	pa	52 (SP)	148
Oxazole-30	pa	25 (Tb), 6.6 (SN)	102
	cm	1.6	
RWJ-56110	pa	0.34 (Tb), 0.16 (SN)	25
	pb	0.44	
RWJ-58259	pa	0.37 (Tb), 0.11 (SN)	94
Nonpeptide antagonists			
1-Phenylacetyl-4-(6-guanidohexanoyl)-piperazine	pa	~1000 (SP)	149
Eryloside F	pa	0.39 (SN), 7.8 (Tb)	83
FR-71113	pa	0.29 (Tb)	96
SCH-79797	pa	0.30 (haTP), 3.0 (Tb)	24
	pb	0.070[a]	
SCH-203099	pa	0.015 (haTP), 0.7 (Tb)	24, 95
	pb	0.045	
SCH-328725	pa	0.27 (haTP) 0.60 (Tb)	97, 98
	pb	0.065	
SCH-73754	pa	0.1-0.3 (haTP, Tb)	UD
	pb	0.015[a]	UD

Agonist used for activating platelet aggregation is in parentheses. Abbreviations: pa = human platelet aggregation; pb = PAR1 binding to human platelet membranes; ra = thymidine incorporation in rat astrocytes; smc = calcium transient of human coronary artery smooth muscle cells; UD = unpublished data; Tb = thrombin; cm = CHRF membranes; SN = SFLLRN; SP = SFLLRNP; SA = SFLLRNPA; SF = SFLLRNPNDKYEPF; N-*trans*-cinnamoyl(p-F)-FpGuFLR-NH_2 = BMS-197525; haTPAP = high-affinity TRAP, {A(p-F)FRChaHarY-NH_2}.

induced proliferation of human aortic smooth muscle cells (105). Of interest is that AFLARAA, unlike the more selective PAR1 antagonists such as RWJ-56110 and SCH-79797, could persistently inhibit aggregation of human platelets induced by a high concentration (22.5 nM) of thrombin. This result, taken together with previous observations (24, 61) of activation of human platelet PAR1 and PAR4 at high concentrations of thrombin, indicates the potential inhibition of both PAR1 and PAR4 by this peptide.MSRPACPNDKYE (T1) was identified as a PAR1 peptide antagonist using phage display (106). Although FLLRNP and T1 were reported to be weakly active in a platelet aggregation assay (106, 107), they show minimal affinity for PAR1 in the haTRAP binding assay. These data and a previous report (86) indicate that T1 is not a PAR1 tethered ligand antagonist. BMS-200261 was the most potent earlier inhibitor of TRAP-stimulated platelet aggregation but exhibited much weaker inhibitory activity against thrombin-stimulated platelet aggregation (31, 86) for some unknown reason. BMS-197525 had an IC_{50} value of 10-30 nM in radioligand binding assays and respective IC_{50} and K_b values of 0.2 and 0.27 μM in platelet aggregation and smooth muscle calcium mobilization assays (31; unpublished data). Recently, BMS-197525 was shown to have partial activity at PAR2 as well as PAR1 in HEK cells (86). A series of azole-based carboxamides, designed after SFLLR, were synthesized (102). The most potent oxazole compound (**30**) inhibited PAR binding with an IC_{50} value of 1.6 μM and gave IC_{50} values of 25 and 6.6 μM against α-thrombin and

SFLLRN-induced platelet aggregation, respectively. Its selectivity was not reported.

RWJ-56110 and its analogues are peptidomimetic PAR1-selective antagonist, based on an indole template. RWJ-56110 showed submicromolar IC_{50} values in inhibiting thrombin- and SFLLRN-induced platelet aggregation as well as calcium mobilization in various smooth muscle and endothelial cells (25). However, it was much weaker (IC_{50}, 3 μM) in inhibiting thrombin-stimulated proliferation of rat aortic smooth muscle cells possibly due to its instability and proteolytic degradation during the long incubation time. Interestingly, RWJ-56110 inhibited internalization of PAR1 induced by thrombin in rat smooth muscle cells (25). This result indicates that PAR1 cleavage is not sufficient and tethered ligand binding is also required for internalization of PAR1. Similar results have been obtained with SCH-79797, a nonpeptide PAR1 antagonist in human vascular smooth muscle cells. A second-generation indazole-based series was developed from RWJ-56110 by altering the guanidine-containing side chain and introducing an indazole template (94). RWJ-58259 showed similar PAR1 selectivity and inhibitory activity to RWJ-56110 but an improved *in vivo* cardiovascular safety profile. However, it may be difficult to develop orally active antagonists from peptidomimetic compounds because of their higher molecular weight and vulnerability to proteolytic degradation.

Nonpetide small molecular antagonists

Eryloside F, a novel penasterol disaccharide possessing potent PAR1 antagonist activity, was isolated from an extract of the marine sponge *Erylus formosus* (83). This compound inhibited thrombin- and SFLLRN-induced platelet aggregation with IC_{50} values of 0.3 and 6 μg/ml, respectively. However, this compound also inhibited platelet aggregation induced by U-46619 with an IC_{50} value of 1.7 μg/ml, indicating poor selectivity for PAR1 over thromboxane A_2 receptor. Another nonpeptide synthetic PAR1 antagonist, FR-17113, selectively inhibited PAR1-mediated platelet aggregation without inhibiting ADP- or collagen-induced platelet aggregation. However, its PAR1 selectivity over other PARs was not reported.

SCH-79797 and SCH-203099 represent the first selective nonpeptide, small-molecular-weight PAR1 antagonists reported (24, 95). SCH-79797 is a pyrroloquinazoline compound and SCH-203099 is an *N*-methyl derivative of SCH-79797. These two compounds show a very similar inhibitory profile. SCH-79797 and SCH-203099 inhibit binding of a high-affinity thrombin receptor activating peptide ([^{3}H]haTRAP) to PAR1 on human platelet membranes with K_i values of 22 and 35 nM, respectively (24). Scatchard analysis of the effect of SCH-79797 on saturation binding of [^{3}H]haTRAP to platelet PAR1 indicates that this compound is a competitive antagonist of the tethered ligand binding sites. SCH-79797 and SCH-203099 inhibited haTRAP-induced aggregation of human platelets with IC_{50} values of 300 and 150 nM, respectively (95). They are less active in inhibiting platelet aggregation induced by α-thrombin than haTRAP, with IC_{50} values of 3000 and 700 nM, respectively (Table II) (24). One of the reasons why these compounds are less active in the platelet aggregation assay than in the binding assay is due to the difference in BSA concentrations used in these assays. Because of avid binding of these compounds to BSA, their apparent inhibitory activity tends to be lower at the higher BSA-containing medium such as that used in the aggregation assay. Unlike haTRAP-induced aggregation, thrombin-induced aggregation was transiently inhibited by SCH-79797 and SCH-203099, probably due to either the slow activation of a second thrombin receptor such as PAR4 or ADP release (24, 61, 68). In contrast, peptidomimetic antagonists such as RWJ-56110 (25) showed persistent (> 5 min) inhibition of platelet aggregation induced by low concentrations of thrombin. The reason for this apparent difference in inhibitory action between the SCH compounds and RWJ antagonists is not known except for the different types of platelet preparations used, *i.e.*, washed *versus* gel-filtered platelet preparations.

In contrast to its effects on platelets, SCH-79797 inhibited thrombin- and TK-induced calcium transients equally well with respective K_i values of 85 and 55 nM in human coronary artery smooth muscle cells (HCASMC). These data indicate that SCH-79797 effectively competes with thrombin-generated tethered ligand or an exogenously added agonist peptide for binding to PAR1 in HCASMC. SCH-79797 and SCH-203099 also inhibited thrombin- and TK-stimulated thymidine incorporation in a concentration-dependent manner and completely at high concentrations in these cells (Table II) (24). In contrast, a peptidomimetic antagonist BMS-197525 (101) only partially inhibited PAR1 selective agonist peptide TK-induced thymidine incorporation in HCASMC. This partial inhibition by a peptide-based antagonist is not surprising when considering the long assay time and rapid degradation of BMS-197525 and PAR1 agonist peptides in the absence of an aminopeptidase inhibitor, amastatin. Complete inhibition of the thrombin-induced calcium transients and thymidine incorporation by SCH-79797 indicates that the thrombin stimulation of calcium transients and thymidine incorporation is mediated by PAR1 in these cells. This shows the usefulness of a selective PAR1 antagonist in clearly establishing whether a particular thrombin effect is mediated via PAR1.

SCH-79797 also blocks thrombin-stimulated MAP kinase phosphorylation as determined by a Western blot analysis as well as thrombin- and TK-induced internalization of PAR1 in human coronary artery smooth muscle cells. SCH-203099 at 1 μM effectively inhibits both haTRAP- and thrombin-stimulated P-selectin expression in human platelets. Both compounds show an inhibitory selectivity for PAR1 over PAR2 and PAR4, as determined by a calcium transient assay and did not inhibit human platelet aggregation induced by γ-thrombin, ADP or collagen (24). SCH-203099 does not inhibit proteolytic activity

of thrombin since cleavage of an N-terminal FLAG-tagged PAR1 by thrombin was not inhibited by 10 μM of the compound. Unlike some peptidomimetic antagonists (31, 101), SCH-203099 did not exhibit any PAR1 agonist activity at concentrations below 10 μM, as determined by a surface P-selectin assay. This compound also shows minimal PAR1 activating effect in a calcium mobilization assay with HCASMC.

Taken together these data indicate that SCH-79797 and SCH-203099 are potent, selective and pure PAR1 antagonists. SCH-79797 appears to bind to and dissociate from PAR1 on human platelets slowly. It requires 60 min of incubation to have full activity of 1 μM of SCH-79797 and 20 min of washing to remove most of the bound compound (24). Cell type-related differences are observed concerning response to gamma-thrombin and hence sensitivity to PAR1 antagonists. While γ-thrombin activates platelet aggregation via PAR4, which was not inhibited by SCH-79797, it stimulated mitogenesis via PAR1 in HCASMC. The latter effect of thrombin was inhibited by SCH-79797.

The second series of Schering-Plough's antagonists are benzimidazole derivatives (97). A representative compound is SCH-328725 that has a K_i of 65 nM in the haTRAP-binding assay. This compound inhibited thrombin- and haTRAP-stimulated calcium transients with IC_{50} values of 0.27 and 0.6 μM, respectively. Unlike the abovementioned pytroloquinazoline compounds, this compound inhibited thrombin- and haTRAP-induced platelet aggregation with a similar potency. SCH-328725 shows about 70-fold selectivity for PAR1 over PAR2 when tested for its inhibitory effect on the PAR1 agonist (TFLLRNPNDK)- and PAR2 agonist (SLIGKV)-stimulated calcium transients in HCASMC. Similar to pyrroloquinazoline compounds, SCH-328725 slowly binds to and dissociates from PAR1 with a dissociation half-time of about 30 min. Examination of concentration-dependent effects of thrombin on calcium transients in the presence of several different concentrations of SCH-79797 and SCH-328725 reveals two different mechanisms: a competitive inhibition of thrombin action at low concentrations and a noncompetitive inhibition at high concentrations of compounds (Fig. 3). This observation may result from slow dissociation of compounds from PAR1 under the non-equilibrium condition.

The third series of PAR1 antagonists are derivatives of the natural product himbacine (97, 100). One of the representative compounds in this series is SCH-73754. SCH-73754 has a K_i value of 12 nM in the platelet PAR1 binding assay and inhibits thrombin- and haTRAP-stimulated platelet aggregation with IC_{50} values of submicromolar concentrations (C. Foster, unpublished data). This class of compounds are highly selective for PAR1 over PAR2 and PAR4, and do not inhibit platelet aggregation induced by ADP and collagen. Human and rat PAR1 show a significant difference in the tethered ligand binding sites (N-terminal site and ECL2), raising the possibility for differential sensitivity to PAR1 tethered ligand antagonists. The peptidomimetic compound RWJ-56110 shows an equal inhibitory activity against rat and human

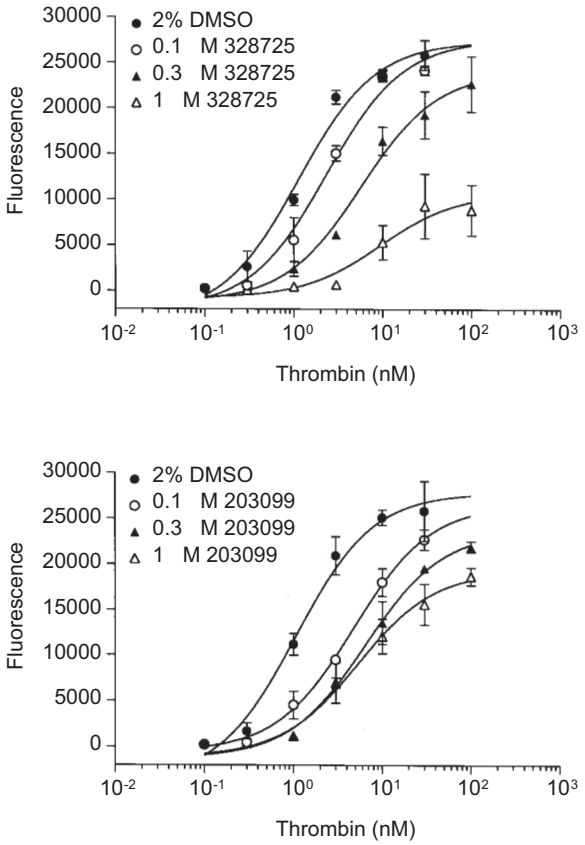

Fig. 3. Effects of SCH-203099 and SCH-328725 on thrombin stimulation of calcium transients in human coronary artery smooth muscle cells. Cells were preincubated with varying concentrations of antagonist for 10 min prior to addition of increasing concentrations of thrombin.

smooth muscle cell PAR1 as examined by a calcium transient assay. A 5- to 10-fold selectivity for human PAR1 is observed with BMS-197525 and SCH-79797. In marked contrast with RWJ-56110, an analogue of SCH-73754 shows a 175-fold selectivity for human *versus* rat PAR1.

Effects of PAR1 antagonists on thrombosis and restenosis

Vascular injury associated with angioplasty procedure results from both thrombosis and restenosis. Although earlier studies demonstrated inhibitory effects of direct thrombin inhibitors on platelet-dependent thrombus formation (1, 108), it was not known whether the protection was mediated through inhibition of the hemostatic action or the cellular action of thrombin. Several lines of evidence suggest that thrombin, acting through PAR1, contributes to thrombosis and restenosis in animal models and in patients after angioplasty procedures (79, 80, 109-111). PAR1 is upregulated in vascular smooth muscle cells in response to vascular injury in animal models (110, 112) and in atherosclerotic plaques from human arteries

but not in normal arteries (109). Also, concentrations of thrombin are increased at the arterial surface after denuding injury and remain elevated for up to 10 days (112). Both activated thrombin and its precursor, prothrombin, can be detected within the neointima of human atherosclerotic lesions (112).

In vivo studies aimed at elucidating the role of PAR1 were carried out employing diverse means of antagonizing PAR1 such as PAR1 antagonist peptides, PAR1 antisense oligonucleotides and antibodies to PAR1. Antisense PAR1 oligodeoxynucleotide did not inhibit intimal hyperplasia following vascular injury in the rabbit (113). However, synthetic antagonist peptides of the PAR1 tethered ligand inhibited platelet-dependent thrombus formation in baboon models by preventing thrombin's cleavage of the platelet PAR1 (79). Similarly, antibodies against PAR1 inhibited thrombus formation in the African green monkey (80) as well as neointimal formation after balloon injury in the rat, without significantly altering the hemostatic parameters (114). More recently, AFLARAA, a PAR1 antagonist peptide, has been shown to inhibit arterial thrombosis induced by electric current in rabbit carotid artery (115). Overall, these data generated with PAR1 antibodies or peptide antagonists support a role of PAR1 activation in thrombogenesis in various animal thrombosis models. They also indicate that PAR1 antagonists may be effective antithrombotics. However, there is some uncertainty in the interpretation of these results due to the potential lack of PAR1 specificity of these agents.

Another approach to defining the role of PAR1 in vascular injury response and restenosis employed genetically modified PAR1 knockout mice. Neointimal and medial areas are decreased in PAR1-deficient mice after carotid artery denuding injury (114), suggesting that the loss of PAR1 signaling protects against restenosis. The latest study has investigated the antithrombotic effects of a potent peptidomimetic PAR1 antagonist, RWJ-58259, in two thrombosis models of guinea pigs and in a rat model of vascular injury. The two thrombosis models are the arteriovenous (A-V) shunt assay monitoring thrombus weight and the Rose Bengal intravascular photoactivation assay monitoring time to occlusion. Administration of RWJ-58259 did not inhibit thrombus formation in either thrombosis models, although local intrashunt delivery in the A-V shunt model elicited a modest antithrombotic effect (116). The poor antithrombotic effects of the PAR1 antagonist were attributed to the presence of PAR1, PAR3 and PAR4 in guinea pigs which, unlike primates, provide two thrombin receptors sensitive to low concentrations of thrombin. In contrast with its minimal effect on thrombosis, perivascular administration of RWJ-58259 significantly reduced neointimal thickness in a vascular restenosis model involving balloon angioplasty in rats. These data demonstrated an important role for PAR1 in vascular injury in this species (116). Since rat platelets, in contrast to those in primates, do not contain PAR1 and are activated by thrombin in a PAR1-independent manner, this finding provides evidence that thrombin contributes to vascular lesion formation through its direct action on vascular cells. However, nonprimates are a poor model for human thrombosis for the following reasons. Nonprimate platelet PAR content and reactivities are different from human platelets (4, 25). In addition, a poor correlation in drug effects was observed between the rodent model of balloon catheter injury and vascular restenosis postangioplasty in humans (117). Further studies with an orally active PAR1 antagonist in a nonhuman primate, whose platelet PAR profile is similar to that of humans, are necessary to more reliably predict the antithrombotic and antirestenosis efficacy of selective PAR1 antagonists in humans.

Therapeutic potential of PAR1 antagonists

Inflammatory diseases

Coagulation is fundamental for hemostasis and is an integral part of inflammatory reactions. Inflammatory mediators promote coagulation by stimulating expression of procoagulant molecules on endothelial cells and macrophages.

1) Glomerulonephritis

Glomerulonephritis (GN) is the most common cause of end stage renal failure. Crescentic GN is a severe and rapidly progressive form of the disease, characterized by glomerular inflammatory cell infiltration, fibrin deposition and local upregulation of procoagulant molecules. Studies of human crescentic GN have shown that local activation of the extrinsic coagulation pathway is important in the pathogenesis of glomerular injury (118). Glomerular depostion of fibrin was thought to be responsible for some of the injurious effects of extrinsic pathway activation. However, recent studies have indicated that fibrinogen-independent effects may also be involved (119). Downregulation of PAR1 associated with upregulation of mRNA (120) in human crescentic GN suggests that the activation of PAR1 may play a role in this disease. Indeed, hirudin, a selective thrombin antagonist, reduced glomerular crescent formation, T cell and macrophage infiltration, fibrin deposition and elevated serum creatinine, which are prominent features of GN (121). To distinguish the procoagulant actions of thrombin from its PAR1-mediated effects, the development of crescentic GN in wild-type and PAR1 knockout mice was compared. PAR1-deficient mice (PAR1–/–), which have normal coagulation, also showed significant protection from crescentic GN compared with wild-type mice. TRAP administration significantly augmented both histological and functional renal injury in wild-type mice developing crescentic GN (121). These results indicate that activation of PAR1 by thrombin or TRAP amplifies crescentic GN and that thrombin has proinflammatory, PAR1-dependent effects that augment inflammatory renal injury.

2) Rheumatoid arthritis

One of the most striking pathological features of rheumatoid arthritis (RA) is the abundant extravascular fibrin depostion within the arthritic joints, indicating ongoing coagulation. Emerging evidence strongly indicates interactions between coagulation and inflammation. Some coagulation factors, besides their well-known role in the coagulation cascade, have been shown to have direct cellular effects (7, 122). Furthermore, recent *in vitro* studies have shown that the PAR1 signaling pathway is proinflammatory as evidenced by PAR1-mediated fibroblast and endothelial cell proliferation as well as activation of inflammatory cells such as monocytes, neutrophils and lymphocytes (7). On the other hand, the end product of the coagulation cascade, fibrin, has proinflammatory effects. The latest study has examined the effect of the thrombin inhibition on collagen-induced arthritis (CIA) in mice, a well-established model of human RA. PEG-hirudin exerted both a preventive effect and curative effect in CIA (123). PEG-hirudin treatment significantly reduced fibrin depostion and also led to decreased levels of synovial PAR1 mRNA (123). These results suggest that PEG-hirudin exerts a beneficial effect in CIA through inhibition of both hemostatic and cellular action of thrombin. CIA exhibits many similar pathologic changes to crescentic GN: activation of coagulation cascade, fibrin deposition and thrombin receptor activation (121, 123). Interestingly, PAR1 inhibition rather than reduction of fibrin deposition protected from crescentic GN as shown by experiments with PAR1–/– mice (122).

3) Lung fibrosis

Thrombin receptor activation was also implicated in pulmonary fibrosis (124) and acute lung injury (125). Higher thrombin concentrations were found in bronchoalveolar lavage fluid from systemic sclerosis patients than from normal controls (124). Thrombin induced smooth muscle-α actin, rapid collagen gel contraction and PKCε through activation of PAR1 (126). Treatment with an antisense oligonucleotide to PKCε or a PKC translocation inhibitor abolished thrombin-induced collagen gel contraction in normal lung fibroblasts. In scleroderma lung fibroblasts PKCε and smooth muscle-α actin were already activated, indicating persistent activation of PAR1 in the scleroderma patients (126). These results suggest that thrombin promotes, via activation of PKCε, differentiation of normal lung fibroblasts to a myofibroblast phenotype that resembles the phenotype observed in scleroderma lung fibroblasts. Chronic presence of thrombin after microvascular injury in lung promotes phenotypic change of normal lung fibroblasts to myofibroblasts in scleroderma patients. These findings indicate the therapeutic potential of PAR1 antagonists in treating interstitial lung fibrosis associated with scleroderma.

4) Radiation-induced enteropathy

Microvascular endothelium within the intestinal mucosa appears to be the actual target of radiation injury (127-129). Radiation induces multiple changes in microvascular endothelium, affecting permeability, vasomotor properties, leukocyte migration and the thrombohemorrhagic imbalance. Radiation induces a marked, chronic deficiency of the natural anticoagulant, thrombomodulin, thrombohemorrhegic imbalance and upregulation of PAR1 in the endothelium of intestinal wall microvasculature (127, 128). Hirudin, a direct thrombin inhibitor, significantly ameliorates intestinal radiation fibrosis and reduces radiation-induced TGF-β1 overexpression (127).

Spinal cord injury

Traumatic and spinal cord injury (SCI) is a leading cause of death and disability. Several recent studies strongly suggest that apoptosis contributes to neuronal cell death in SCI and a variety of neurodegenerative disorders (130, 131). Nerve injury induces a burst of thrombin activity in mouse sciatic nerve (132). SCI initiates a thrombin-triggered cascade of events following activation of PAR1, culminating in increased calcium influx, caspase activation and death of motor neurons and other cells in the spinal cord (130, 133). Intraperitoneal administration of recombinant soluble thrombomodulin (70 μg) greatly improved recovery after SCI in rats (134). Taken together, these data suggest a possible beneficial effect of a PAR1 antagonist in SCI.

Cancer

The close association between cancer and coagulopathies has long been recognized in humans. Thrombin-catalyzed, crosslinked fibrin formation is a characteristic histopatholgical finding in many human and experimental tumors. Increased thrombin generation was detected in tumor cells and host cells (*e.g.*, macrophage-derived), while PAR1 was highly expressed in tumor cells, invasive cell lines and in breast carcinoma biopsy specimens (6). Expression of PAR1 is closely correlated with metastatic propensity of breast cancer cells (135). Thrombin promotes tumor cell adhesion to endothelial cells, subendothelial matrix, fibronectin and von Willebrand factor under static conditions and platelet-dependent adhesion to endothelial cells under flow conditions (6). Enhanced expression of $\alpha_{IIb}\beta_3$ (gpIIb/IIIa) and other glycoproteins (*e.g.*, P-selectin) mediates tumor adhesion. Thrombin promotes the invasion of aggressive breast tumor cells (136) and migration of human transitional-cell carcinoma J82 (137). Thrombin is also implicated in the development of experimental pulmonary metastasis (138). Since many tumor cells contain more than one type of PAR (6, 135), it was not known if PAR1

plays a role in thrombin-stimulated metastasis. Evidence that PAR1 is the primary receptor for thrombin comes from a recent study on primary cultures obtained from two human glioblastoma samples (139). Calcium transients induced by thrombin and $TRAP_{1-6}$ were completely blocked by the thrombin receptor antagonist peptide T1. Furthermore, another study has shown that PAR1 antisense cDNA was able to inhibit breast carcinoma invasion in the Matrigel *in vitro* invasion assay (140). PAR1 increases the invasive properties of tumor cells primarily by increased adhesion to extracellular matrix components. While PAR1 overexpressing cells were invasive *in vitro*, invasion was further enhanced by ligand activation of PAR1 (141). The application of anti-$\alpha_v\beta_5$ antibodies specifically attenuated this PAR1-induced invasion, indicating cooperative involvement of this integrin. However, several reports showed that thrombin or a PAR1 activating peptide could inhibit migration and invasion of breast cancer cells and several tumor cell lines (135, 142).

The Rho proteins are critical mediators of many transforming proteins including Ras (143). The search for novel oncogenes from a cDNA library from the B6SutA(1) mouse myeloid cell line has identified PAR1 as a transforming gene (144). Although inhibitors of thrombin failed to block PAR1 transforming activity, a PAR1 mutant that cannot be cleaved by thrombin was nontransforming, indicating the necessary role of PAR1 activation. In this situation, a PAR1 antagonist may still block transformation regardless of the type of protease activating PAR1 since it will block the tethered ligand binding site. PAR1's transforming activity was blocked by coexpression of dominant negative RhoA and $G\alpha_i$. These observations suggest that PAR1-induced growth transformation is mediated in part by activation of RhoA. Taken together, these data suggest an important role of PAR1 in tumor metastasis, transformation and the potential utility of PAR1 antagonists in the treatment of cancers. PAR2 also appears to play a role in the regulation of some forms of cancer (6).

PAR1 antagonists *versus* other antithrombotic agents

It is desirable to compare PAR1 antagonists with other antithrombotic therapeutic agents on the market. Potential advantages and disadvantages of PAR1 antagonists over thrombin inhibitors, gpIIb/IIIa antagonists and ADP ($P2Y_{12}$) antagonists are given below.

Advantages

Orally available PAR1 antagonists may be developed which have a minimal bleeding side effect unlike the other available antithrombotic agents. Attempts to develop orally active thrombin inhibitors and gpIIb/IIIa antagonists have not yet been successful. Their therapeutic window is very narrow.

PAR1 tethered ligand antagonists, but not thrombin inhibitors, can block platelet and other cell PAR1 activation induced by factor Xa and other proteinases such as a granzyme.

Unlike thrombin inhibitors, PAR1 antagonists do not prevent thrombin from activating the anticoagulant pathway, allowing continued activation of protein C, a very important anticoagulant and antiinflammatory protein. Infusion of activated protein C has been found to protect animals and humans from septic shock.

PAR1 antagonists, but not gpIIb/IIIa or ADP antagonists, have a direct antiproliferative action on vascular smooth muscle cells and may have potential to block restenosis after angioplasty.

PAR1 antagonists only partially block gpIb mediated signaling and platelet binding to von Willebrand factor in the subendothelial matrix whereas thrombin inhibitors and ADP antagonists may cause complete inhibition of gpIb signaling. Complete blockade of gpIb may cause prolonged bleeding, as evidenced by von Willebrand's disease and Bernard-Soulier syndrome.

Disadvantages

Thrombin but not PAR1 antagonists will block all protease-activated receptors for thrombin (*e.g.*, PAR1 and PAR4 in human platelets).

Long-term therapy with a PAR1 antagonist after angioplasty might have an adverse effect on the stability of thrombotic plaques because of its antiproliferative activity. However, the expected anticoagulant and antiinflammatory effects of PAR1 antagonists might counter the above effect.

Conclusions

Thrombin, the central molecule in blood coagulation, also has profound effects on virtually every aspect of vascular wall biology, including regulation of vessel tone, SMC proliferation, differentiation and migration, endothelial activation, vascular development, atherogenesis and angiogenesis. Biochemical properties of PAR1 activation mechanism, intracellular signaling, desensitization and functional responses have been rather well characterized in the past 10 years. Scientists in this field have begun to investigate the role of PAR1 in normal function as well as the pathophysiology of thrombosis, restenosis and inflammatory diseases using various methods of inhibiting PAR1 including neutralizing antibodies, knockout and PAR1 antagonists. From these and earlier studies, PAR1 appears to be a main thrombin receptor and plays an important role in cardiovascular and inflammatory diseases.

Major progress has been made in the development of PAR1 antagonists, and several selective PAR1 antagonists have already been reported. PAR1 selective antagonists and PAR knockout mice will play invaluable roles in defining PAR1 function in the cell type of interest in view

of the known species and cell type-related differences and the presence of multiple PARs in the same cell. Antagonists of other PARs also need to be developed to facilitate defining the role of an individual PAR. We are now entering a golden era of the pharmacology of PARs and the prospect of therapeutic agents emerging from research and development in the field of PAR1 in the next 10 years is good.

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