Published in final edited form as:

Trends Pharmacol Sci. 2017 August ; 38(8): 701–716. doi:10.1016/j.tips.2017.05.001.

Targeting PAR1: Now what?

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Abstract

Protease-activated receptors (PARs) are a ubiquitously expressed class of GPCRs that enable cells to respond to proteases in the extracellular environment in a nuanced and dynamic manner. PAR1 is the archetypal family member and has been the object of large-scale drug development programs since the 1990s. Vorapaxar and drotrecogin-alfa are approved PAR1-targeted therapeutics, but safety concerns have limited clinical use of vorapaxar and questions regarding the efficacy of drotrecogin-alfa led to its withdrawal from the market. New understanding of mechanisms of PAR1 function, discovery of improved strategies for modifying PAR1 function, and identification of novel indications for PAR1 modulators have provided new opportunities for therapies targeting PAR1. This review will critically evaluate prospects for the next generation of PAR1-targeted therapeutics.

Keywords

Protease-activated receptor 1; vorapaxar; activated protein C; pepducins; parmodulins

PAR1 Mechanism of Action

Protease-activated receptor (PAR) 1 is the prototypical member of a family of G-protein coupled receptors (GPCRs) that are activated by a variety of proteases. Thrombin was the first protease shown to cleave PAR1; indeed, PAR1 was originally known as the thrombin receptor. Coughlin and colleagues identified PAR1 as a thrombin substrate using an expression cloning strategy, showing that PAR1 possesses a thrombin cleavage site in its N-terminus [1]. While the vast majority of GPCRs are stimulated by the association of soluble small molecules or peptides to deep binding pockets on the extracellular face of the receptor, PAR1 is activated when cleavage at its N-terminus exposes a tethered ligand that interacts with a shallow binding site [1,2]. This intramolecular activation mechanism produces a conformational change that is transmitted to cognate G proteins and β -arrestin (Figure 1) [3–7]. This section describes the different proteases that cleave PAR1, how cleavage by

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Conflict of Interest: RF holds a patent describing parmodulin 1 (JF5) and parmodulin 2 (ML161). Trends Box

different proteases results in stimulation of distinct signaling pathways, and how PAR1 interacts with other PARs present on cell surfaces.

Protease-mediated activation

Following the identification of PAR1 as a substrate for thrombin, proteases of increasing diversity were found to cleave the extracellular domains of PAR1. These proteases can be sorted into those that activate signaling through PAR1 and those that inactivate the receptor (Figure 1) [8]. Serine proteases remain the largest class of proteases to cleave PAR1. The canonical cleavage site on the extracellular face of PAR1 for thrombin is PR⁴¹^ SFLLRN⁴⁷ (Figure 2). Mutational studies suggest that the tethered ligand interacts with extracellular loops on the superficial face of the receptor rather than in a deep binding pocket [2]. In contrast to thrombin, activated protein C (APC) is a serine protease that cleaves PAR1 at R46 (PR⁴¹SFLLR⁴⁶^ NPNDKYEPF) [9]. As described below, activation of PAR1 by the tethered peptide formed from APC results in very different cellular responses compared with those activated by thrombin. Matrix metalloproteases MMPs including MMP1, MMP2, MMP9, MMP13 also cleave PAR1 (Figure 1) [8,10–12], albeit at different sites compared to serine proteases (Figure 2). Inactivating proteases cleave PAR1 distal to activation sites, thereby preventing stimulation (Figure 1, Figure 2). Overall, proteolysis of PAR1 by different proteases and at distinct cleavage sites enables a single receptor to orchestrate entirely different and even opposing cellular responses.

In addition to cleaving at unique sites, different proteases interact with PAR1 by distinct mechanisms. Thrombin binds via exosite I to a hydrophobic hirudin-like sequence DK⁵¹YEPF⁵⁵ on the PAR1 extracellular face, facilitating the interaction of the PAR1 cleavage site with active site of thrombin [13,14]. This cleavage mechanism is very efficient (e.g., approximately 10,000-fold more potent than that of APC) [15]. Following cleavage of PAR1 by thrombin, the receptor serves as a protease-responsive guanine nucleotide exchange factor, replacing the GDP of associated G α subunits with GTP. G α_q , G $\alpha_{12/13}$, and G α_i have all been shown to couple with PAR1 (Figure 1). APC mediated cleavage is different and best studied in endothelial cells. Briefly, APC first binds to the endothelial cell PC receptor (EPCR) [16], which is compartmentalized in lipid rafts and orients the protease on the endothelial surface [17–19]. Binding of APC to EPCR also promotes recruitment of G protein coupled receptor kinase 5, which phosphorylates the cytoplasmic tail of PAR1 [17–20], facilitating recruitment of β -arrestin 2 [5]. Not only do thrombin and APC interact with PAR1 via distinct mechanisms, these proteases also elicit discrete downstream signaling pathways.

Biased signaling

Among the most remarkable features of PAR1 is its ability to stimulate opposing signaling pathways depending on the proteolytic stimulus. Thrombin and other proteases that strongly activate PAR1-mediated guanine nucleotide exchange elicit marked cellular activation. In platelets, thrombin-mediated activation of PAR1 results in aggregation, granule release, activation of adhesion proteins, and profound shape change. In endothelium, PAR1 activation stimulates exocytosis, expression of adhesion proteins, loss of barrier function, transcriptional activation and angiogenesis. Neurons, smooth muscle cells, immune cells,

epithelial cells, and fibroblasts among others all undergo substantial phenotypic changes following thrombin-mediated activation of PAR1. While signaling pathways differ among the various cell types, certain themes in early PAR1-mediated signaling cascades are known. PAR1-mediated GDP-GTP exchange in Gαq enables activation of phospholipase C isoforms, resulting in hydrolysis of PIP₂, with the generation of inositol triphosphate (IP₃) and diacylglycerol (DAG). IP₃ acts at the IP₃ receptor to facilitate calcium release from intracellular stores, which contributes to the activation of multiple downstream signaling proteins. DAG stimulates protein kinase C isoforms. PAR1-mediated activation of Gα_{12/13} stimulates RhoGEF, with downstream activation of the Rho-Rho kinase pathway and myosin light chain. PAR1-mediated Gα_i activation inhibits adenylyl cyclase. Mapping of these canonical signaling pathways have now been augmented with phosphoproteomics, which has revealed over 2000 phosphosites in endothelium, many on proteins not previously recognized as components of the PAR1 signaling cascade [21]. Functional outputs of PAR1 pathways vary substantially between cell types but in the most general terms include promotion of cell growth, stimulation of adhesive properties, granule release and cytoskeletal reorganization, with stress fiber formation at cell-cell junctions.

Activation of PAR1 by APC stimulates a very different set of cellular responses. In endothelial cells, these responses can be characterized as anti-inflammatory, anti-apoptotic and barrier protective [22]. PAR1-mediated cytoprotective can be stimulated in many cell types, including neurons, dendritic cells, astrocytes, epithelial cells, fibroblasts, and others. Cellular signaling responses to APC are complex and PAR1 is not the only relevant substrate mediating its cytoprotection (for reviews see [22,23]). However, knockdown or inhibition of PAR1 blocks cytoprotective effects in many preclinical disease models [22]. APC-mediated cleavage elicits phosphorylation of the PAR1 cytoplasmic tail and recruitment of β-arrestin and dishevelled-2 [5,19], leading to Rac1 and Akt activation. These pathways result in fortification of cell-cell barriers and inhibition of NF-κB-mediated transcriptional activation [5,9,16,24,25].

Transactivation

While intracellular signaling induced by PAR1 can activate other receptors in an indirect transactivation process (reviewed in [26]), physical interactions of PAR1 with other PARs enables direct transactivation [27]. This allows PAR1 to influence signaling through other PARs and, along with signaling bias, adds to the complexity and versatility of PAR1 signaling. Direct transactivation occurs with heterodimer formation between PAR1 and PAR4. Thrombin bound to PAR1 within the heterodimer is capable of 'reaching over' and cleaving PAR4, thereby stimulating activation of calcium flux in platelets [28]. On endothelium, formation of a PAR1-PAR2 heterodimer regulates PAR2 function. In this case, thrombin associated with PAR1 generates a tethered ligand that can transactivate PAR2 at the PAR2 ligand binding site [29], switching signaling in endothelium from RhoA to Rac1 [30]. PAR1 can also bind PAR3, which acts as an allosteric modulator of PAR1 [31]. The possibility of targeting the interaction of PAR1 with other PARs to interfere with heterodimer signaling has been proposed [32], although small molecules that specifically target direct transactivation have not yet been designed.

Pharmacological Modulators of PAR1

The unique activation mechanism of PAR1 creates several challenges for drug development. Soluble molecules targeting the ligand binding site must block a tethered ligand that has an effective concentration of 400 μM . Binding of orthosteric inhibitors must be nearly irreversibly (e.g., vorapaxar) to compete with the tethered ligand. Furthermore, the biased nature of pathways downstream of PAR1 raises the concern that inhibition of deleterious signaling could also interfere with cytoprotective signaling. Modulation of PAR1 using proteases (e.g., recombinant APC, aka drotrecogin- α) is complicated by the fact that all PAR1 targeted proteases have alternative substrates, creating significant off-target considerations.

Variation in PAR1 tissue distribution among different species also complicated drug development. In particular, most non-primate species including mice, rats, dogs, and rabbits express PAR3 and PAR4 on their platelets, but not PAR1 [33,34]. Although these species do express PAR1 on other tissues [35]. Species that express PAR1 and PAR4 on their platelets include humans, monkeys, and guinea pigs [36]. However, while guinea pig models have been used to assess PAR1 antagonists, guinea pig platelets express PAR3 along with PAR1 and PAR4, potentially complicating use of guinea pigs for antiplatelet agents [37]. These species differences limit the use of rodent studies in the development of PAR1 antagonists.

Despite these obstacles drug development efforts over the past decade have led to the identification of many PAR1 agents, some of which have progressed to clinical studies whereas others have been used as tools to understand PAR1 signaling. Here, we describe several agents, including peptide-based agents, small molecules, and therapeutic proteases used to modulate PAR1 activity (Figure 3; for an overview see Table I), with a focus on molecules that are most promising for clinical applications.

Peptide-based Agents

The synthetic peptide SFLLRN-NH₂, which corresponds to the tethered ligand sequence unmasked by thrombin cleavage, functions as a soluble PAR1 agonist that can activate the receptor independent of proteolysis. Early strategies to develop PAR1 inhibitors focused on designing peptides and peptidomimetics based on this tethered ligand sequence. Lead compounds BMS-200261 [38], a NH₂-acyl tetrapeptide developed by Bristol-Myers Squibb, and RWJ-58259 [39], an indazole-derived peptide generated by Johnson Pharmaceuticals, both inhibited PAR1-mediated platelet aggregation *in vitro*. BMS-200261 treatment was protective in a middle cerebral artery model of ischemic stroke [40,41]. RWJ-58259, the most potent peptidomimetic *in vitro*, reduced thrombosis in a non-human primate model, but failed to show any effect in guinea pigs [42]. Despite the use in many animal models, none of these peptidomimetics advanced to clinical trials, mainly due to their limited efficacy in animal models.

To identify peptide-based PAR1 inhibitors with increased potency, Covic et al. used a novel strategy targeting the intracellular loops of PAR1 that interact with cognate G proteins [43,44]. They created pepducins, a class of cell-penetrating lipidated agents. Pepducins are comprised of a peptide whose sequence corresponds to a region in PAR1 intracellular loops

i1, i2, or i3 covalently linked to a lipid moiety (e.g. palmitate). The lipid moiety facilitates transport through and anchoring in the lipid bilayer. Close proximity of the peptide to the GPCR-G protein interface interferes with G protein-mediated signaling. Unexpectedly, the first PAR1 i3-derived pepducin (P1pal-19) activated platelet calcium flux and aggregation in a manner similar to the PAR1 agonist peptide SFLLRN. Truncation of peptide sequences showed an association between length and pepducin activity, with shorter pepducins (P1pal-7; P1pal-12[S]) exerting antagonist activity, whereas longer ones (P1pal-19; P1pal-13) exhibited agonist activity in platelet aggregation assays [43–45]. Pepducins are highly selective for their receptor and have been designed for many other GPCR's (see review [45]). But how do pepducins modulate receptor activity? The mechanism remained unclear until a report by Zhang et al. [46]. The P1pal-19 agonist pepducin was shown to induce a PAR1 'on'-conformation by interacting with the PAR1 H8 helix in the cytoplasmic tail and a (D/N)PXXYYY motif on TM7. As P1pal-19 induces a PAR1 'on'-conformation, one might expect that inhibitory pepducins would stabilize PAR1 in an 'off'-conformation thus blocking G-protein mediated signaling. Indeed, P1pal-7 (also known as PZ-128) exposure induces an inactive receptor conformation [47]. It is likely that different pepducins induce an array of conformations. Elucidating how members of the pepducin family stabilize or disrupt PAR1 conformation and differentially modulate G-protein coupling and downstream signaling could inform development of new therapeutics with more nuanced control of PAR1 signaling.

Studies have shown that PAR1 pepducins (for full list see Table I) are not just effective modulators of GPCR activity *in vitro* but also in animal models of sepsis [30], cancer [48] and thrombosis [47] (see Table for overview). Inhibition of PAR1 using PZ-128 was associated with protection in a FeCl3 arterial thrombosis model [47]. This pepducin reversibly suppressed PAR1-mediated platelet aggregation and arterial thrombosis in guinea pigs and baboons, without any effect on bleeding [47]. A small Phase I safety and efficacy study was conducted for the use of PZ-128 in the prevention of acute thrombotic applications of percutaneous coronary interventions [49]. PZ-128 dose-dependently inhibited SFLLRN-induced platelet aggregation. Inhibitory activities were rapid and reversible by high concentrations of SFLLRN, with no effects on bleeding or coagulation. Although this is the first pepducin to be evaluated in clinical studies, the short half-life (1.3–1.8 hours), lack of oral availability and need for continuous infusion suggest that it will be best used in the hospital setting. In addition, treatment with higher doses of PZ-128 was associated with allergic reactions. Phase II safety studies in a larger population of patients with percutaneous coronary intervention are underway (see Table II).

Small Molecules

High throughput screening of compound libraries for platelet PAR1 inhibitors yielded several small molecules antagonists including SCH79797 [50], FR171113 [51], F16618 [52], E5555 (*atopaxar*) [53], and SCH530348 (*vorapaxar*) [54] (for overview see Table II). Although antithrombotic effects in animal models were observed for all these molecules, only vorapaxar and atopaxar were evaluated in large-scale clinical trials. These two compounds were notable for their high oral bioavailability, superior potency and selectivity. Vorapaxar and atopaxar are competitive inhibitors that bind at the tethered ligand site and

compete with the endogenous agonist. Atopaxar is a bicyclic amidine derivate developed by Eisai Inc. that showed inhibitory effects in a rabbit hemorrhage model [55] and a guinea pig photochemically-induced thrombosis model [56]. However, in Phase II trials performed in patients with acute coronary syndrome (LANCLOT-ACS) [57] or chronic coronary artery diseases (LANCLOT-CAD) [58], atopaxar use was associated with transaminitis and QT prolongation at higher drug doses as well as with increased major bleeding. Hence, atopaxar never advanced to Phase III clinical trials.

Vorapaxar, discovered by Schering-Plough and developed by Merck, is a synthetic tricyclic 3-phenylpyridine based on the natural alkaloid himbacine [59]. The compound is so specific for human PAR1 that it does not even block mouse PAR1. Randomized, placebo-controlled trials that were conducted in healthy subjects [60] and in patients undergoing percutaneous coronary intervention (PCI) [61] showed that vorapaxar is safe and well-tolerated, and did not increase clinically significant bleeding. Given these results and a trend in reduction of myocardial infarction, two pivotal phase III clinical trials were initiated. TRACER studied 12,944 patients treated with usual care (aspirin and/or P2Y12 inhibition) with or without vorapaxar in patients with ACS (i.e., primary prevention) [62]. The primary endpoint was cardiovascular death. After two years, the Data and Safety Monitoring Board closed out the trial after finding a 3.4-fold increase in intracranial hemorrhage (ICH) without any beneficial effect. TRA 2°P TIMI 50 studied 26,449 patients with known cardiovascular disease treated with usual care with or without vorapaxar (i.e., secondary prevention) [63]. This study demonstrated a reduction in cardiac death from 12.4% in the usual care arm compared with 11.2% in the vorapaxar arm. A 2-fold increase in ICH was observed. Based on TRA 2°P, vorapaxar was approved by the FDA in 2014 for secondary prevention of thrombotic events in patients with a history of MI or CAD, with exclusion of patients with a history of transient ischemic attack or stroke. Co-administration of vorapaxar with other anti-platelet agents might contribute to increased bleeding events as improved safety and efficacy was observed in vorapaxar groups not receiving aspirin and/or thienopyridine [62]. Currently multiple clinical trials using vorapaxar are ongoing (see Table II for a full list of clinical trials). Such studies will enable us to assess the use of PAR1 antagonism for other indications.

Once bound essentially irreversibly to PAR1, orthosteric inhibitors such as vorapaxar interfere with both deleterious and cytoprotective downstream pathways. Allosteric modulation, as evidenced with pepducins, enables selective control of downstream signaling pathways. Using high-throughput screening of a small molecule library for inhibitors of PAR1-mediated platelet granule secretion, a novel class of PAR1 modulators that act at the cytosolic face of PAR1 were identified [64–66]. These small molecules, termed parmodulins, selectively block some G-protein signaling pathways ($G_{\alpha q}$) but not others ($G_{\alpha 12,13}$), whereas orthosteric inhibitors block all downstream pathways. Perhaps more importantly, parmodulins 1 (aka JF5) and 2 (aka ML161) block PAR1-mediated platelet and endothelial activation, without interfering with APC-protective signaling in endothelial cells [66]. Parmodulins inhibit thrombus formation *in vivo* without prolonging bleeding [66]. By simultaneously blocking pathological signaling and activating protective signaling, these compounds could provide an alternative strategy to treat complex inflammatory disorders. Currently, these compounds are used to assess proof-of-principle in preclinical studies.

Current parmodulins lack oral availability and their pharmacokinetics are unknown. However, efforts to develop orally available parmodulins with improved pharmacological profiles are underway.

Proteases

Recombinant APC (drotrecogin alfa) received FDA approval for the treatment of severe sepsis in 2001 [67]. Although APC was originally thought to benefit septic patients by virtue of its anticoagulant activity alone, Riewald et al. published in 2002 that APC activated a cytoprotective genetic program in endothelium in a PAR1-dependent manner [16]. Over the next decade, a substantial literature evolved showing that cleavage of PAR1 by APC was protective in a wide range of cell-based and *in vivo* disease models [22]. Yet despite these laboratory findings, there were questions of the efficacy of drotrecogin-alfa in the setting of severe sepsis that lead European Medicines Agency to ask that Eli Lilly reproduce the findings of the PROWESS trial. This second trial (PROWESS-SHOCK) [68] (see Table 2) failed to show any benefit and drotrecogin-alfa was withdrawn from the market in 2011.

Since the anticoagulant effect of APC is a bleeding liability that limits its dosing and therefore its efficacy, investigators generated cytoprotection-selective variant that were just as protective as APC in pre-clinical models. These signaling-selective APC variants were constructed to have limited anticoagulant activity by mutating N-linked glycan sites and/or residues in APC important for interaction with FVa or Protein S: 3K3A-APC (KKK191-193AAA), RR229/230AA, 5A-APC (KKK191-193AAA+ RR229/230AA), RD222/237CC, K193E, D36A/L38D/A39, L38D and L38D/N329Q [22,69–71]. The non-anticoagulant forms of APC stimulated PAR1-mediated cytoprotective signaling *in vitro* and exerted beneficial effects in murine models of sepsis and ischemic stroke [22,69–71]. As cytoprotective-selective variants circumvent bleeding problems associated with APC's anti-coagulant function, these variants are promising agents for clinical applications. 3K3A-APC is currently the only variant that has advanced to clinical trials. High-dose bolus injection of the 3K3A-APC variant was found safe in healthy subjects in a Phase I clinical study [72]. A Phase II trial is ongoing (NCT02222714) to assess the safety and efficacy in ischemic stroke patients (see Table 2).

Indications for PAR1 modulation

With many new agents engineered using novel strategies to circumvent the limitations of previously developed PAR1 modulations, a current challenge is to match an agent with an optimal indication. The opportunity is great, since PAR1 has been implicated in such a large number of physiological and pathophysiological processes. Abundant indications, however, can complicate the planning of a drug development pathway. In the broadest terms, new agents are targeted for either (1) inhibition of PAR1 prothrombotic, proinflammatory, tumor promoting activities, and profibrotic activities or (2) activation of PAR1-mediated cytoprotective pathways.

Inhibition of PAR1 signaling

Cardiovascular—The size of TRACER and TRA 2°P enabled subgroup analysis to assess vorapaxar in specific populations of patients with cardiovascular disease. In patients from TRA 2°P with peripheral vascular disease, vorapaxar reduced hospitalization as a result of acute limb ischemia (2.3% with vorapaxar, 3.9% with placebo, $P=0.006$) and peripheral artery revascularization (18.4% with vorapaxar, 22.2% with placebo, $P=0.017$). Bleeding was more frequent in patients taking vorapaxar (7.4% with vorapaxar, 4.5% with placebo, $P=0.001$) [73]. Diabetics participating in the TRA 2°P trial demonstrated additional benefit with regard to the primary endpoint of cardiovascular death, MI, or cerebrovascular accident (12.6% with vorapaxar, 15.7% with placebo, $P=0.004$). There was a 1.59-fold increase ($P=0.02$) in bleeding in diabetic patients receiving vorapaxar compared with the control group [74]. Coronary artery bypass grafting (CABG) is frequently complicated by ischemia, thought to be related to increased thrombin generation. Vorapaxar significantly reduced ischemia compared to placebo with a hazard ratio of 0.55 ($P=0.005$). Bleeding was not significantly increased in patients undergoing CABG who received vorapaxar [75]. These studies underscore the fact that not all indications for antiplatelet therapy are equal and PAR1 inhibition may be more effective in some antiplatelet niches than others. Several clinical trials either planned or underway evaluate vorapaxar in vascular indications such as AV fistula maturation for hemodialysis access, testing in patients with diabetes mellitus, lower extremity bypass grafts, and peripheral arterial disease.

Preventing tumor progression—PAR1 was identified as an oncogene based on its ability to transform fibroblasts more than 20 years ago [76]. Since that time, PAR1 has been shown to participate in several aspects of malignancy including angiogenesis, tumor invasiveness, metastases and migration. Pharmacological inhibition or genetic deletion of PAR1 blocks growth of several cancer types in culture and impairs progression of several tumor types in mice, including lung cancer [48], melanoma [77], colon cancer [78] and pancreatic cancer [79]. Breast cancer has been most actively studied. Activation of PAR1 via MMP or thrombin promotes breast cancer progression [80], while pharmacological inhibition or genetic knockdown of PAR1 impairs viability [81]. Ectopic expression of functionally active PAR1 in breast cancer cells, when injected into mice, induces a hormone-refractory invasive phenotype that metastases, whereas cells expressing a non-signaling PAR1 do not develop invasive breast cancer [82]. In a xenograft model of breast cancer, progression was markedly attenuated following infusion of a pepducin, P1pal-7, with taxotere compared to taxotere alone. Metastases were inhibited by P1pal-7 alone [81]. A tumor-targeted insertion peptide coupled to intracellular loop 3 of PAR1 also blocks breast cancer growth [83]. PAR1 is selectively overexpressed in patients with high grade infiltrating ductal breast carcinoma compared to lower grade disease [84]. Similarly, PAR1 expression was negatively correlated with survival in patients with HER2-negative breast carcinoma [85].

Several additional potential indications for PAR1 antagonism are suggested by results in animal models. Inhibition or deletion of PAR1 prevents fibrosis in several cell culture-based and animal models of fibrosis including liver [86], heart [87], and lung [88]. Colitis induced in an experimental model was markedly decreased in PAR1 knockout mice or in mice

exposed to the PAR1 antagonist RW-56110 compared with control and expression of PAR1 was upregulated in intestinal biopsies from patients with colitis [89]. Administration of FR171113 decreased intestinal vagal apoptosis observed following experimentally induced colitis [90]. PAR1 functions in the immune response to viral infection and PAR1 knockout is protective in mouse models of influenza and coxsackievirus-induced myocarditis [91,92]. A human study to evaluate PAR1 inhibition using vorapaxar in controlled endotoxemia is ongoing (NCT02875028, Table II).

Stimulating PAR1 cytoprotective signaling

Stimulation of cytoprotective signaling mediated by PAR1 is beneficial in a wide range of preclinical models from sepsis to amyotrophic lateral sclerosis (see [22], Tables I and II). Earlier studies were performed with rAPC. However, since rAPC was withdrawn from the market, studies with APC mutants that lack anticoagulant activity have become more prevalent.

Sepsis—Recombinant APC was the first agent shown to stimulate PAR1-mediated cytoprotection approved for human use (in severe sepsis) [93]. It is important to recall, however, that rAPC was initially developed for sepsis based on its anticoagulant properties and was later found to stimulate PAR1 cytoprotection [16]. The PAR1-independent anticoagulant effects of rAPC lead investigators to develop novel APC mutants (discussed above) that maintain the ability to signal through PAR1, but have negligible anticoagulant activity. Both APC and these novel mutants act through PAR1 to provide cytoprotection in a wide range of conditions [22].

Stroke—In light of the complication of intercranial hemorrhage with vorapaxar, it is interesting that PAR1 agonists are being evaluated for use in stroke. There is abundant work in neuronal cells demonstrating that stimulation of PAR1 cytoprotective signaling is beneficial in models of neuronal death [94,95]. In a mouse model, APC protected against brain hemorrhage in a PAR1-dependent manner [96]. An APC mutant (3K3A-APC) that lacks anticoagulant activity and acts solely by stimulation of PAR1-mediated cytoprotective has been tested in preclinical models of ischemic stroke [69]. More recently, 3K3A-APC was shown to stimulate restoration neuronal repair by neuronal stem cells transplanted into brains following ischemia [97]. 3K3A-APC is currently in clinical trials as a neuroprotectant in the setting of ischemic stroke.

Wound healing—PAR1 is present on keratinocytes and has been reported to function in wound healing. Several case reports and small series suggest efficacy of APC in chronic skin ulcers in human subjects [98–101]. In one study, 12 diabetic patients with non-healing ulcers were randomized to either topical APC or saline for 20 weeks [101]. APC-treated wounds showed a decrease in affected area, reduced inflammation, and increased vascular proliferation. While a larger study is required to truly determine efficacy of APC in this setting, the application of topical APC for wound healing speaks to the range of potential indications for APC and creates applications for using it.

Many other potential indications for stimulation of cytoprotection through PAR1 via APC or APC mutants have been proposed. PAR1 is expressed in neuronal and non-neuronal cells (e.g., astrocytes and microglia) within the CNS. APC and 3K3A-APC have been shown to be beneficial in animal models of amyotrophic lateral sclerosis [102] and neurogenesis [97], respectively. Evidence of beneficial PAR1-mediated cytoprotection in CNS may be suggested from extremely rare side effects of vorapaxar such as diplopia [103] and amyotrophic lateral sclerosis [104], if these prove to be *bona fide* adverse effects of PAR1 inhibition. PAR1-mediated protective signaling by rAPC and signaling-selective APC variants are protective in preclinical models of ischemia reperfusion injury evaluating reperfusion injury in heart, brain, liver, and kidney [22]. APC also prevents remote acute lung injury following intestinal ischemia reperfusion injury [105,106]. APC and/or signaling-selective APC mutants improve organ function in several models of transplantation, are protective in the setting of radiation exposure, and decrease band formation following surgery [22,107,108]. Successful development of 3K3A-APC or another signaling-selective APC could enable the targeting of PAR1-mediated cytoprotection in a wide range of clinical application.

Concluding Remarks

The initial challenge for the development of PAR1 antagonists was identifying molecules that bound tightly enough at the ligand binding site to compete with the tethered ligand. Achievement of this goal ultimately bought FDA approval of the first-in-class PAR1 inhibitor, vorapaxar. The new challenge for PAR1 antagonists is to provide improved or similar efficacy compared with vorapaxar, without the bleeding risk. Two strategies to achieve this objective have been identification of agents with new mechanisms of action (e.g., pepducins, parmodulins) and identification of new, perhaps niche, indications for PAR1 antagonists. Specific indications will be dictated in large part by agent characteristics such as pharmacokinetics, oral availability, reversibility, and toxicity.

The challenges for therapeutic modulation of PAR1 cytoprotection are to demonstrate that activation of this pathway is clinically important and that it can be achieved without non-PAR1-mediated complications of proteases (e.g. bleeding caused by APC). APC mutants that lack anticoagulant activity are likely to pose little bleeding risk. Despite dozens of animal models suggesting a role for cytoprotection in disease models, clinical efficacy has not yet been established. Proof of efficacy will be reliant on choosing an optimal clinical application. Sepsis, while a critically important indication, is challenging for single agents. Testing of 3K3A-APC in stroke is an exciting development that may be an easier strategy for providing clinical proof of the importance of the PAR1 cytoprotective pathway.

Current work in the PAR1 field suggests several strategies for accomplishing the clinical development of novel PAR1 modulators and addressing outstanding questions in the field (see Outstanding Questions Box). Foremost is the use of full data sets that include cell-based, animal models, and human studies to inform decisions for clinical development. Human studies derive not only from the invaluable information from studying more than 40,000 patients in the vorapaxar trials, but also studies evaluating PAR1 expression (e.g., breast cancer) and potentially PAR1 gene variants in disease. For use in complicated,

refractory process such as sepsis and IRI, it will be useful to consider combination therapies that target known contributory pathways in addition to those downstream of PAR1. PAR1 inhibition could be more effective when used in tandem with other modalities such as extracorporeal blood cleansing devices [109] or additional reagents that target endothelium, such as Tie2 agonists [110]. Beyond therapeutics that are already in or are primed for clinical trial, strategies that leverage the growing understanding of PAR1 biased signaling could impact therapy. Agents that simultaneously stimulate cytoprotective signaling and block detrimental signaling could demonstrate improved efficacy for some indications. Current research evaluating PAR1 signaling bias has provided tractable endpoints for high throughput screens selecting for biased PAR1 modulators. Such modulators may act at the ligand binding site, but more likely, will target the cytoplasmic face of the receptor as do pepducins and parmodulins.

Acknowledgments

This work was funded by grants from NHLBI (HL125275, HL112809, and T32 HL007917). The authors would like to acknowledge the significant contributions of investigators in the PAR1 field whose substantial contributions were not included in this review owing to space limitations.

Glossary

Activated protein C (APC)

a serine protease that circulates in blood and exerts anti-coagulant effects, by inactivating coagulation factor Va and factor VIIIa. It also has cytoprotective effects on cells by cleaving PAR1 and eliciting downstream signaling

Allosteric Modulator

a substance that affects receptor function by binding at a site distinct from the ligand binding site

Biased receptor signaling

when a ligand demonstrates functional selectivity by stimulating (or inhibiting) a particular signal transduction pathway, but not others

Cytoprotection

a signaling mechanism that protects cells against harmful agents. In endothelial cells, this includes stimulation of anti-inflammatory and anti-apoptotic activities as well as maintenance of barrier function

Drogetocin alfa

a recombinant form of activated protein C

G-protein coupled receptors (GPCRs)

seven-transmembrane domain receptors that mediate signaling through G proteins on the cytoplasmic face of the receptor. GPCRs constitute the largest class of surface receptors, are expressed in all tissues, and respond to a wide variety of extracellular stimuli

Matrix metalloproteases (MMPs)

zinc-dependent proteases that cleave matrix proteins, process bioactive proteins, and activate cell surface receptors

Orthosteric antagonist

an antagonist that acts at the ligand binding site

Parmodulins

a class of small molecules that act at the cytosolic face of PAR1 and selectively block some G-protein signaling pathways but not others

Pepducins

a class of lipidated peptides that modulate PAR1 function by acting at the cytosolic face of PAR1

Phases of drug development

Pre-clinical: testing of the efficacy and toxicity of a drug *in vitro* and in animal models.

Phase I: Evaluation of safety of a drug on healthy volunteers.

Phase II: Evaluation of safety and efficacy of a drug on a small group of patients.

Phase III: Evaluation of efficacy and side effects on a large patient population.

Phase IV: Evaluate the drug's long-term safety and effects in the population after FDA approval.

Protease-activated receptor-1 (PAR1)

the prototypical member of the PAR family of GPCRs, which are activated by a unique mechanism involving proteolytic cleavage. It is also known as the thrombin receptor

Serine Protease

a large class of proteases with an active site that includes a catalytic triad consisting of three amino acids, a nucleophile (serine) a base (often histidine), an acid (often aspartic acid). They function to cleave peptide bonds in a wide variety of substrates

SFLLRN

a synthetic PAR1 agonist peptide that can activate PAR1 independent of proteolysis

Signaling-selective APC variants

mutant APCs designed to impair APC anticoagulant properties, but maintain the ability to cleave PAR1 and activate PAR1-mediated cytoprotective signaling

Thrombin

a serine protease that circulates in blood and serves an essential role in clotting (by converting fibrinogen to fibrin) and activating platelets (by cleaving PAR1)

Vorapaxar

a small molecule inhibitor of PAR1 that is approved by the FDA

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Trends Box

- Two agents targeting PAR1, recombinant APC and vorapaxar, are FDA approved. However, their use has been limited by questions of efficacy and safety, respectively.
- Study of PAR1 signaling bias has demonstrated the extent to which PAR1 stimulation can elicit deleterious versus cytoprotective signaling depending on the activating protease and the physiological context.
- New pharmacological approaches including rationally engineered, signaling-selective APC mutants and agents that target the cytosolic face of PAR1 have been developed.
- Human studies evaluating novel indications for approved PAR1 modulators and testing new pharmacological approaches are currently underway.

Outstanding Questions Box

- Will the widespread distribution of PAR1 translate into new indications for PAR1 inhibition beyond blockade of platelet activation? Or will this distribution lead to unwanted side effects?
- Will the cytoprotective effects of PAR1 signaling so amply demonstrated in preclinical models translate into significant efficacy in a clinical setting (e.g., stroke)?
- Is inhibition of PAR1-mediated cytoprotection using orthosteric antagonists an unavoidable consequence of this mechanism of inhibition and, if so, is it clinically relevant?
- Can targeting the cytosolic face of PAR1 enhance the control of downstream signaling and simultaneously block deleterious signaling while stimulating cytoprotective signaling?

Figure 1. Overview of protease sensing via PAR1

PAR1 (red) is a GPCR that can be either stimulated (green circle) or inactivated (red circle) by proteolytic cleavage mediated either by either serine proteases (blue) or matrix metalloproteases (gold). For stimulating proteases, cleavage of an N-terminal segment reveals a tethered ligand (yellow) that associates with a shallow binding site on the extracellular face of the receptor. Ligation of the binding site induces a conformation change in the receptor that is transmitted to cognate G-proteins or β -arrestin. Protease agonists that stimulate cytoprotective signaling include APC and signaling-selective APC variants that lack anticoagulant activity, such as 3K3A-APC. Orthosteric inhibitors that act at the ligand binding site include vorapaxar and atopaxar. Allosteric modulators that act at the cytosolic face of the receptor include pepducins and parmodulins.

Figure 2. Protease cleavage of the N-terminus of PAR1

The amino acid sequence of protease sensitive regions within the N-terminus of PAR1 is shown. Cleavage sites for representative serine proteases (*blue*) and matrix metalloproteases (*gold*) cleavage sites are indicated with arrowheads.

Figure 3. Mechanism of action of different PAR1 modulators

A) Vorapaxar (*blue*) is an orthosteric inhibitor that binds nearly irreversibly to the ligand binding pocket on the extracellular surface of PAR1 (*red*). **B)** 3K3A-APC (*black*) is a signaling-selective APC mutant that lacks anti-coagulant activity. It binds to the endothelial protein C receptor (*green*) on the endothelial surface prior to cleaving PAR1. Its cleavage of PAR1 elicits cytoprotective signaling. **C)** Parmodulin 2 (*blue*) is a small molecule that act at the cytosolic surface of PAR1. It interferes with some, but not all, downstream signaling pathways and elicits cytoprotective signaling in endothelium. **D)** PZ-128 (aka P1pal-7, (*blue*) is a pepducin consisting of seven amino acids derived from the il3 loop of PAR1 linked to palmitate to facilitate cell permeability and anchor the peptide in the membrane. PZ-128 interacts with the cytosolic face of the receptor and stabilizes its inactive conformation.

Table 1

PAR1 modulators that have been evaluated in animal models.

Agent	Class	Site of Action	In vivo activity	Ref.
RWJ-58259	peptide	Ligand binding site	Antithrombotic in a non-human primate thrombosis model. Inhibited restenosis in a rat balloon angioplasty model.	[37][111]
BMS-200261	peptide	Ligand binding site	Protective in a middle cerebral artery occlusion (MCAO) model of stroke and a mouse model of Parkinson's disease.	[35][36]
P1pal-7 (PZ-128)	Lipidated peptide	i3	Inhibition of tumor growth in breast, ovarian and lung cancer xenograft mouse models. Antithrombotic activity in guinea pig and baboon models of thrombosis.	[42][43][81][80]
P1pal-10S	Lipidated peptide	i3	Inhibition of tumor growth in a xenograft mouse model of lung cancer.	[43]
P1pal-12	Lipidated peptide	i3	Inhibition of lung fibrosis in a bleomycin-induced pulmonary fibrosis mouse model.	[112]
P1pal-12S	Lipidated peptide	i3	Time-dependent effects on mortality, vascular leakage and disseminated intravascular coagulation (DIC) in mouse models of sepsis.	[30]
P1pal-13	Lipidated peptide	i3	Time-dependent effects on mortality, vascular leakage and DIC in mouse models of sepsis.	[30]
Vorapaxar	Small molecule	Ligand binding site	Inhibition of platelet aggregation ex vivo in rat and cynomolgus monkey models.	[54]
Atopaxar	Small molecule	Ligand binding site	Inhibitory effects in a rabbit model of hemorrhage and a guinea pig model of photochemically-induced thrombosis.	[50][51]
SCH79797	Small molecule	Ligand binding site	Antithrombotic in rat models of arterio-venous shunt and myocardial/kidney ischemia reperfusion injury. Maintains vascular integrity in subarachnoid hemorrhagic rat model.	[113][114][115]
F16618	Small molecule	Site unknown	Antithrombotic in arterio-venous rat shunt model and carotid thrombosis guinea pig model. Inhibited restenosis in a rat balloon angioplasty model.	[47][116][117]
FR-171113	Small molecule	Site unknown	Antithrombotic in a FeCl ₃ carotid artery thrombosis model in guinea pigs.	[118]
Parmodulin 1 (JF5)	Small molecule	Cytosolic face	Inhibition of platelet thrombus formation in murine laser-injury thrombosis model.	[6]
Parmodulin 2 (ML161)	Small Molecules	Cytosolic face	Inhibition of platelet thrombus formation in murine laser-injury thrombosis model.	[61]
3K3A-APC	Protease	Cleavage site	Reduction in mortality in sepsis mouse models. Improved functional outcome and reduced infarct size in murine models of embolic and ischemic (MCAO) stroke. Promotes neurogenesis with functional engraftment of stem cells in murine stroke.	[119][120][97]
5A-APC	Protease	Cleavage site	Reduction in mortality in mouse models of sepsis.	[121]
APC-K193E	Protease	Cleavage site	Protective in a mouse model of myocardial ischemia/reperfusion injury.	[122]
APC-L38D	Protease	Cleavage site	Protective in a mouse ischemia/reperfusion injury model of ischemic stroke.	[66]
APC-L38D/N329Q	Protease	Cleavage site	Protective in a mouse ischemia/reperfusion injury model of ischemic stroke.	[66]
APC-D36A/L38D/A39V	Protease	Cleavage site	Protective in a mouse ischemia/reperfusion injury model of ischemic stroke.	[65]

■ Orthosteric antagonists – ■ Allosteric antagonists – ■ Allosteric agonist – ■ antagonists – ■ proteases

Table II

Clinical trials of PAR1 modulators that are either approved or in development.

Intervention	Identifier No. Status, Phase	Study	N	Condition	Results/Outcome measures	Ref
Vorapaxar	NCT02394730 Recruiting Phase I/II	Treatment of HIV-associated inflammation and coagulopathy ADVICE	~60	HIV	<i>Primary:</i> Change in D-dimer levels. <i>Secondary:</i> CD4+ and CD8+ counts. HIV RNA copies, BARC bleeding episodes.	NA
Vorapaxar	NCT00132912 Completed Phase II	Safety and efficacy in addition to standard care <i>TRA-PCI</i>	1030	Non-urgent PCI	Well-tolerated; no increase in TIMI bleeding even upon concomitant treatment with aspirin and clopidogrel.	[61]
Vorapaxar	NCT00684203 Completed Phase II	Safety and efficacy in addition to standard care in Japanese patients	120	ACS, MI Ischemic stroke	Significant reduction in incidence of periprocedural MI. No increase in TIMI major or minor bleeding. X	[123]
Vorapaxar	NCT00526474 Completed Phase III	Safety and efficacy in addition to standard care in patients with history of atherosclerotic disease <i>TRA 2°P-TIMI 50</i>	26449	Heart attack and stroke	Reduction in risk of cardiovascular death and ischemic events. <i>Adverse effects:</i> Increased risk of moderate to severe bleeding, including intracranial hemorrhage (ICH).	[63]
Vorapaxar	NCT00527943 Terminated Phase III	Safety and efficacy in addition to standard care TRACER	12944	ACS	No significant reduction in cardiovascular death. <i>Adverse effects:</i> Significant increase in major bleeding, including ICH which led to termination of the study.	[62]
Vorapaxar	NCT02660866 Recruiting Phase IV	Addition of vorapaxar to standard antiplatelet therapy XLPAD-TRACE	~200	Peripheral artery disease	<i>Primary:</i> Peak walking time on a graded treadmill test. <i>Secondary:</i> Claudication onset time on graded treadmill test.	NA
Vorapaxar	NCT02545933 Recruiting Phase IV	Pharmacodynamic effects in addition to P2Y ₁₂ receptor inhibitors ± aspirin VORA-PRATIC	~126	Myocardial infarction	<i>Primary:</i> Maximal platelet aggregation dual antiplatelet therapy (DAPT) ± vorapaxar. <i>Secondary:</i> Maximal platelet aggregation vorapaxar + prasugrel/ticagrelor versus vorapaxar + standard DAPT.	NA
Vorapaxar	NCT02875028 Recruiting Phase IV	Crossover study in a human endotoxemia model	~16	Healthy subjects	<i>Primary:</i> Changes in prothrombin fragments. <i>Secondary:</i> Platelet PAR1 levels, inflammatory, coagulation, endothelial activation, fibrinolytic biomarkers, body temp, heart rate, blood pressure & counts, oxygen, leukocyte subset.	NA
Vorapaxar	NCT02548650 Recruiting Phase IV	Pharmacodynamic effects in addition to standard dual antiplatelet therapy OPTIMUS-5	~64	MI ± diabetes mellitus	<i>Primary:</i> Max. platelet aggregation vorapaxar ± DAPT or clopidogrel. <i>Secondary:</i> Max. platelet aggregation diabetic vs non diabetic patients with vorapaxar + DAPT	NA
rAPC	Not applicable Completed Phase III	Safety and efficacy PROWESS	1690	Severe sepsis	Reduced mortality. Increased bleeding.	[93]
rAPC	NCT00604214 Completed Phase III	Safety and efficacy PROWESS-SHOCK	1696	Severe sepsis	No significant reduction in mortality.	[68]

Intervention	Identifier No. Status, Phase	Study	N	Condition	Results/Outcome measures	Ref
rAPC	Not applicable Completed Pilot trial	Clinical benefit of topical application on chronic lower leg ulcers	12	Diabetes	Significantly reduced wound area and patient stress scores. Reduced inflammatory cell infiltration and increased vascular proliferation.	[101]
rAPC	NCT00943267 Completed Observational	Bronchial instillation of LPS: Effect on local coagulation and inflammation	~52	Healthy subjects	APC treatment enhanced LPS-induced coagulation and pro-inflammatory responses.	[124]
PZ-128	NCT01806077 Completed Phase I	Safety, tolerability, PK and PD	32	CAD or multiple CAD risk factors	Reversible platelet inhibition. No effects on bleeding, coagulation or ECG parameters.	[49]
PZ-128	NCT02561000 Recruiting Phase II	Safety and efficacy	~600	Non-emergent PCI	<i>Primary:</i> Incidence of bleeding. <i>Secondary:</i> Incidence of major cardiovascular events.	NA
rAPC-3K3A	NCT01660230 Completed Phase I	Safety and pharmacokinetics of single and multiple ascending doses	64	Healthy subjects	Well tolerated and safe at multiple doses with highest dose of 540 µg/kg.	[72]
rAPC-3K3A	NCT022222714 Recruiting Phase II	Safety, PK and preliminary efficacy of multiple doses in combination with tPA ± mechanical thrombectomy RHAPSODY	~100	Ischemic stroke	<i>Primary:</i> Adverse events <i>Secondary:</i> PK, max observed plasma conc. (C _{max}), elimination rate constant, t _{1/2} , total clearance, volume distribution, aPTT versus C _{max} , bleeding in brain.	NA