Challenges and Opportunities in Protease-Activated Receptor Drug Development

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Annu. Rev. Pharmacol. Toxicol. 2017. 57:349–73

First published online as a Review in Advance on September 9, 2016

The *Annual Review of Pharmacology and Toxicology* is online at pharmtox.annualreviews.org

This article's doi: 10.1146/annurev-pharmtox-011613-140016

Keywords

thrombin, platelet, endothelial, biased signaling, dimerization

Abstract

Protease-activated receptors (PARs) are a unique class of G protein–coupled receptors (GPCRs) that transduce cellular responses to extracellular proteases. PARs have important functions in the vasculature, inflammation, and cancer and are important drug targets. A unique feature of PARs is their irreversible proteolytic mechanism of activation that results in the generation of a tethered ligand that cannot diffuse away. Despite the fact that GPCRs have proved to be the most successful class of druggable targets, the development of agents that target PARs specifically has been challenging. As a consequence, researchers have taken a remarkable diversity of approaches to develop pharmacological entities that modulate PAR function. Here, we present an overview of the diversity of therapeutic agents that have been developed against PARs. We further discuss PAR biased signaling and the influence of receptor compartmentalization, posttranslational modifications, and dimerization, which are important considerations for drug development.

INTRODUCTION

G protein–coupled receptors (GPCRs) mediate a myriad of biological processes, and dysregulation of GPCR function is linked to various pathological conditions, making this receptor class an attractive drug target. Most GPCRs are activated by small, natural molecules such as peptides, hormones, or ions that can be mimicked easily by synthetic analogues; accordingly, this receptor class is the largest target for US Food and Drug Administration (FDA)-approved therapeutics. Protease-activated receptors (PARs) are unique members of the large superfamily of GPCRs, transduce cellular responses to extracellular proteases, and are important drug targets. However, the proteolytic nature of PAR activation, which results in irreversible activation, is distinct from most other GPCRs. Owing to this irreversible nature of PAR activation, the development of receptor-specific antagonists has presented an unusual challenge.

Since the discovery of PAR1 in 1991 (1), researchers have made significant progress in understanding not only PAR1 but also other PARs' function in vivo, receptor-signaling properties in various cellular contexts, and the regulation of receptor signaling and function. Despite these advances, the development of pharmacological entities that function as selective orthosteric or allosteric ligands targeting specific PARs has been difficult. Consequently, multiple diverse approaches have been used to develop drugs against PARs. In this review, we discuss the diversity of therapeutic agents that have been developed to target PARs and their clinical efficacy. We further discuss the potential opportunity and challenges in developing effective pharmacological agents targeting PARs, including new perspectives on biased signaling properties of the receptors as well as posttranslational modifications and dimerization, which can influence receptor-specific signaling responses and are important considerations for drug development.

PAR FUNCTION AND ACTIVATION

PARs have important functions in vascular physiology, development, inflammation, and cancer progression. Four PARs are encoded in the mammalian genome. PAR1, the family prototype, transmits cellular responses to thrombin, the key effector protease of the coagulation cascade. PAR3 and PAR4 are also activated by thrombin, whereas PAR2 is activated by trypsin-like serine proteases. PAR1 was the first PAR discovered in a search for a receptor that conferred thrombin responses on human platelets (1). PAR2 was discovered next in a genomic library screen and found to mediate trypsin responses (2). PAR3 and PAR4 were identified subsequently and shown to mediate thrombin signaling in mouse platelets (3–5), indicating that PARs are expressed differentially in distinct cell types in a species-specific manner. PAR1, PAR3, and PAR4 are expressed primarily in various cell types in the vasculature, including platelets, fibroblasts, and endothelial and smooth muscle cells, and are major effectors of thrombin signaling in vivo. However, other proteases can cleave and activate these receptors in a way similar to thrombin or through unique sites in the N terminus (**Table 1**). PAR2 is expressed in vascular, intestinal, and pulmonary cells and primarily mediates inflammatory responses associated with tissue injury. Similar to the other PARs, PAR2 is activated by multiple proteases including trypsin, tryptase, and upstream coagulation factors. Thus, the particular protease that functions as the physiological regulator of PAR activation depends on the tissue and cell type.

The activation of PARs occurs through proteolytic cleavage of the extracellular N-terminal domain, which generates a new N terminus that functions as a tethered ligand and binds to the receptor through an intramolecular interaction to trigger transmembrane signaling (**Figure 1**) (6). Synthetic peptides that mimic the tethered ligand sequence can activate PAR1 independently of thrombin and proteolytic cleavage (7, 8). Thrombin recognizes specific cleavage sites in the N

Table 1 PAR cleavage sites

Receptor	Protease	Cleavage site/tethered ligand	Cellular response
PAR1	Thrombin	ATLDPR$_{41}$/SFLLRNPNDKYEPF	Platelet activation, endothelial barrier permeability
	APC	ATLDPR$_{41}$/SFLLR$_{46}$/NPNDKYEPF	Endothelial cytoprotection, anti-inflammatory
	MMP1	ATLD$_{39}$/PRSFLLRNPNDKYEPF	Breast cancer progression, restenosis
	MMP13	ATLDPRS$_{42}$/FLLRNPNDKYEPK	Ventricular myocyte and fibroblast fibrosis
	Elastase	ATLDPRSFLL$_{46}$/RNPNDKYEPF	Endothelial barrier protection
	Proteinase 3	A$_{36}$/TLDPRSFLLRNPNDKYEPK	Endothelial barrier permeability
PAR2	Trypsin	SSKGR$_{36}$/SLIGKV	Inflammation, hyperalgesia
	Tryptase	SSKGR$_{36}$/SLIGKV	Inflammation, hyperalgesia, mast cell responses
	Factor VIIa, factor Xa	SSKGR$_{36}$/SLIGKV	Endothelial inflammation
	Kallikrein	SSKGR$_{36}$/SLIGKV	Cell proliferation
PAR3	Thrombin	LIPK$_{38}$/TFRGAPPNSFEEFPFS	Murine platelet activation
	APC	LIPKTFR$_{41}$/GAPPNSFEEFPFS	Endothelial cytoprotection
PAR4	Thrombin	PAPR$_{47}$/GYPGQV	Platelet activation
	Cathepsin G	PAPR$_{47}$/GYPGQV	Platelet activation

Abbreviations: APC, activated protein C; MMP, matrix metalloprotease; PAR, protease-activated receptor.

terminus of PAR1, PAR3, and PAR4 (**Table 1**). A second interaction occurs between thrombin's anion-binding exosite I and an acidic hirudin-like sequence in the N-terminal domain of PAR1 and PAR3, resulting in exquisite specificity for thrombin binding and cleavage (6). PAR4 lacks the second binding site and exhibits a low affinity for thrombin (3). Similar to other PARs, trypsin-like proteases recognize and cleave distinct sites in the N terminus of PAR2, resulting in tethered ligand

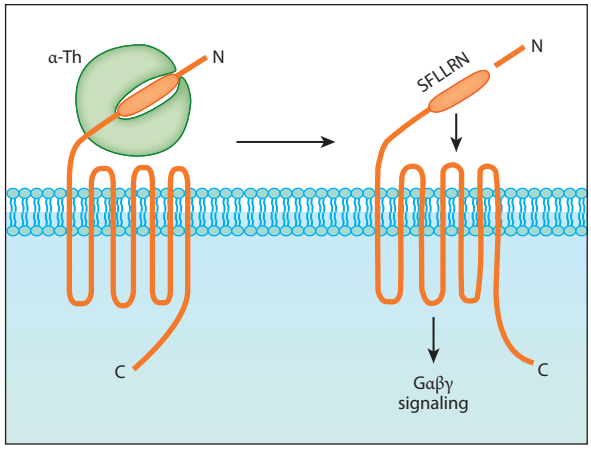

Figure 1

Proteolytic mechanism of protease-activated receptor 1 (PAR1) activation. The mechanism of PAR activation is best understood for PAR1. Thrombin (α-Th) binds to and cleaves the extracellular N terminus of PAR1 at a specific arginine (R) residue located at position 41. This results in the generation of a new N terminus that binds intramolecularly to the receptor to trigger transmembrane signaling mediated by heterotrimeric G proteins comprising α and βγ subunits. Synthetic peptides (SFLLRN) that represent the first six amino acids of the newly formed N terminus can activate PAR1 independently of thrombin and receptor cleavage.

generation and activation (**Table 1**) (2). Once cleaved, activated PARs undergo conformational changes within transmembrane helices that facilitate interaction with heterotrimeric G proteins. PAR1 is promiscuous and interacts with multiple distinct types of these G proteins, including $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12/13}$ (9, 10). PAR2 and PAR4 also appear to interact with $G\alpha_q$ and $G\alpha_{12/13}$ proteins, whereas PAR3 does not appear to signal autonomously, at least in certain cell types. In addition to heterotrimeric G proteins, activated PARs can also signal via interaction with β-arrestins and transforming growth factor β–activated kinase-binding protein-1 (TAB1) (11–13), which function as scaffolds for mitogen-activated protein (MAP) kinase activation. Recent studies showed that PARs display biased agonism. Biased agonism or functional selectivity refers to the capacity of different ligands to stabilize unique active conformations of a GPCR that facilitates activation of distinct signaling responses. Several studies have demonstrated clearly that different proteases cleave PARs at distinct sites that result in the generation of unique tethered peptide ligands that promote signaling to distinct heterotrimeric G protein subtypes or β-arrestins. These findings raise the possibility that biased ligands that selectively activate or block certain signaling pathways induced by PAR activation can be developed and may function as important therapeutic agents.

DIVERSE DRUG DEVELOPMENT FOR PAR1

The only current clinical agent that targets PARs is the PAR1 antagonist vorapaxar. The early observation that PAR1 predominantly mediates thrombin-induced platelet function (14, 15) resulted in two decades of aggressive work toward developing small-molecule PAR1 antagonists and culminated in the approval of vorapaxar by the FDA in 2014 for the prevention of thrombotic cardiovascular events (16, 17). As a result of extensive drug development programs, the quality and quantity of PAR1 antagonists far exceeds those targeting the other members of the PAR family. Indeed, a remarkable diversity of approaches to PAR1 antagonism has been reported.

The earliest PAR1 inhibitors were function-blocking antibodies directed against either the thrombin-binding exosite of the receptor (18–20) or the region spanning the thrombin cleavage site (**Figure 2**) (21). Although effective in vitro and in vivo, a major limitation of this antibody-based approach has been the ease with which the inhibitory activity is overcome at increased agonist concentrations (18, 19, 21), suggesting improvements in K_D values of PAR1 function-blocking antibodies are required. Similarly, a series of peptide and peptidomimetic PAR1 inhibitors were used in early studies of PAR1 function. These agents mimicked the binding of the endogenous tethered ligand sequence of the receptor but contained modifications to prevent subsequent receptor activation (8, 22). Several key experimental reagents that emerged from this approach include a series of NH_2-acyl tetrapeptides, such as BMS-197525 and BMS-200261 (21–23), and the RWJ series of indole and indazole derivatives, such as RWJ-56110 and RWJ-58259 (24–26). Although used widely in early studies investigating PAR1 function, these peptide-based antagonists generally lack specificity, potency, or both, and many function as partial agonists, resulting in very limited current use. Here, we focus on three distinct classes of PAR1 antagonists—small-molecule antagonists, pepducins, and parmodulins—that bind to distinct sites on the receptor (**Figure 2**) and have been used in recent studies.

PAR1 Small-Molecule Antagonists

Several small-molecule PAR1 antagonists have been developed, and two of these, atopaxar and vorapaxar, have been examined in large-scale clinical trials. Both atopaxar and vorapaxar bind reversibly at or near the tethered ligand orthosteric binding site within the second extracellular

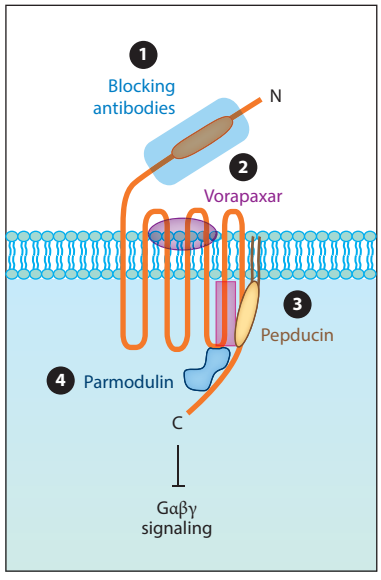

Figure 2

A remarkable diversity of pharmacological agents target PAR1 to block G protein signaling. ❶ PAR1 function-blocking antibodies directed against the N-terminal region have been shown to block receptor signaling. In addition, small-molecule inhibitors such as the FDA-approved vorapaxar ❷ that target the orthosteric binding site block PAR1 signaling and are currently used in the clinic. Two classes of newer molecules—pepducins ❸ and parmodulins ❹—target the intracellular regions of the receptor to inhibit certain G protein signaling pathways.

loop of PAR1 (**Figure 2**) and compete with the endogenous receptor-tethered ligand activation mechanism to function as classical competitive antagonists.

Atopaxar, formerly known as E5555, is a bicyclic amidine derivative with a molecular weight of 609 Da (**Figure 3**). Atopaxar inhibited binding of a tritiated synthetic agonist peptide, [^{3}H]SFLLRN, to PAR1 and inhibited human platelet aggregation in response to thrombin and a PAR1-specific agonist peptide (**Table 2**) (27). In vivo, atopaxar demonstrated antithrombotic activity in several small animal models of thrombosis, including photochemically induced thrombosis in guinea pigs (27). In this model, atopaxar prolonged the time to thrombotic vessel occlusion approximately 2-fold without impacting skin bleeding time significantly. Given the promising in vivo efficacy of atopaxar, human trials were undertaken in patients with acute coronary syndrome (LANCELOT-ACS) and chronic coronary artery disease (LANCELOT-CAD) (28, 29). However, patients taking higher doses of atopaxar exhibited increased liver enzymes, prolonged QT intervals leading to tachyarrhythmia, and increased rates of major bleeding events in Phase II safety trials (28, 30), indicating significant on- and off-target side effects. As a result, atopaxar did not advance to Phase III trials.

Vorapaxar, formally known as SCH530348, is also a reversible, competitive, small-molecule PAR1 antagonist. Vorapaxar is a synthetic tricyclic 3-phenylpyridine analogue of the naturally occurring alkaloid himbacine with a molecular weight of 591 Da (**Figure 3**) (31). Functionally, vorapaxar inhibits aggregation of human platelets induced by thrombin and a PAR1-activating peptide (**Table 2**). Pharmacokinetic profiling of vorapaxar was performed initially in rats and monkeys, where it was shown to have high oral bioavailability and an elimination half-life of

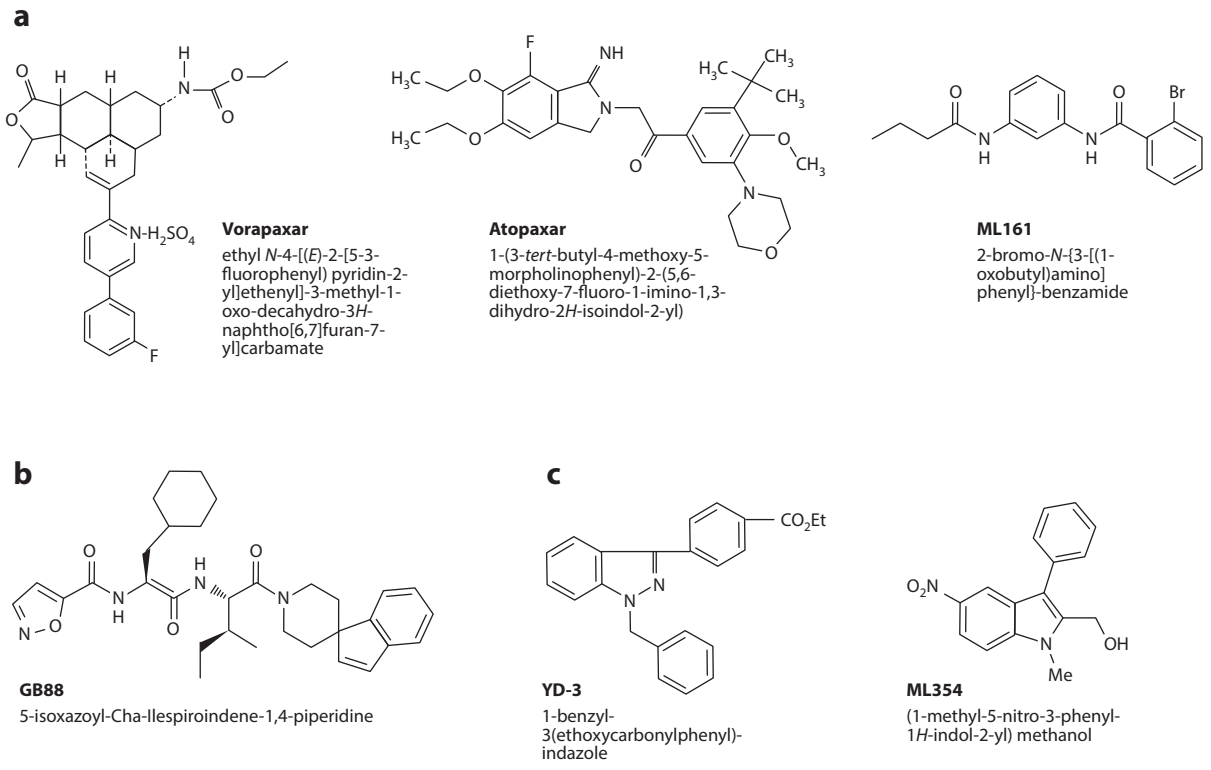

Figure 3

Structures of small-molecule inhibitors that target PARs. (*a*) Small molecules that target PAR1 have distinct structures. Vorapaxar is an analogue of the naturally occurring himbacine (31), atopaxar is a bicyclic amidine derivative (27), and ML161 parmodulin contains a 1,3-diaminobenzene core structure (51). (*b*) The PAR2 small-molecule antagonist GB88 is an N-terminal isoxazole, L-cyclohexylalanine, and L-isoleucine, but with a bulky C-terminal spiroindenepiperidine that confers PAR2 antagonism at low micromolar concentrations (59). (*c*) The YD-3 small-molecule inhibitor of PAR4 is an indazole derivative (68), and ML354 is a recently discovered, substituted indole, PAR4-selective antagonist (78).

13 h (31, 32). In humans, vorapaxar is well tolerated and long lasting, with a terminal plasma half-life of 126–269 h and greater than 90% bioavailability after a single loading dose (33). In contrast to the Phase II clinical trial findings with atopaxar, no abnormalities in liver function have been associated with long-term use of vorapaxar (33, 34). Vorapaxar has been assessed in two large-scale Phase III clinical trials: the Thrombin Receptor Antagonist for Clinical Event Reduction in Acute Coronary Syndrome (TRACER) trial and the Thrombin Receptor Antagonist for Secondary Prevention of Atherothrombotic Ischemic Events (TRA 2°P-TIMI 50) trial (35). Although TRACER failed to reach its primary endpoint (a composite of cardiovascular death, myocardial infarction, stroke, recurrent ischemia, or urgent coronary revascularization), the TRA 2°P-TIMI 50 trial showed significant benefit with vorapaxar use. Specifically, vorapaxar, when administered in combination with standard-of-care antiplatelet therapy, reduced the rate of cardiovascular events from 10.5% to 9.3% with $P < 0.001$ without a significant effect on fatal bleeding. As a result, vorapaxar was approved by the FDA in mid-2014 for the prevention of thrombotic complications in patients with myocardial infarction or peripheral artery disease.

Table 2 Pharmacological properties of leading PAR antagonists

Target receptor	Antagonist	Antagonist class	IC_{50} (assay)	In vivo activity
PAR1	Vorapaxar	Small molecule	25 nM (PAR1-AP–induced human platelet aggregation) 47 nM (thrombin-induced human platelet aggregation) (31)	Single dose of 40 mg inhibits >80% of PAR1-AP–induced human platelet aggregation (33)
	Atopaxar	Small molecule	31 nM (PAR1-AP–induced human platelet aggregation) 64 nM (thrombin-induced human platelet aggregation) (27)	Single dose of 50 mg inhibits >80% of PAR1-AP–induced human platelet aggregation (29)
	PZ-128	Pepducin	0.5 μM (PAR1-AP–induced human platelet aggregation) (49)	2 mg/kg abolishes PAR1-AP–induced human platelet aggregation (48)
	ML161	Parmodulin	2 μM (PAR1-AP–induced calcium release in HUVECs) (51)	5 mg/kg decreased thrombosis size by 73% in murine model of laser-induced thrombosis (51)
PAR2	ENMD-1068	Small molecule	2.5 mM (PAR2-AP–induced calcium release in LLC cells) (138)	4 mg (I.P.) inhibits joint swelling in mice (138) 25 mg/kg (I.P.) inhibits endometriotic lesions (139)
	GB88	Small molecule	2 μM (PAR2-AP–induced calcium release in A549 cells) (59)	10 mg/kg (P.O.) inhibits PAR2-dependent paw edema in rats (59)
PAR4	tcY-NH_2	Peptidomimetic	190 μM (PAR4-AP–induced human platelet aggregation) (140)	Not reported
	P4pal-10	Pepducin	1 μM (PAR4-AP–induced human platelet aggregation) (43)	3 μM increases tail bleeding time in mice (43)
	P4pal-il	Pepducin	0.6 μM (PAR4-AP–induced human platelet aggregation) (67)	0.13 mg/kg in guinea pigs decreases occlusion time after thrombotic injury nonsignificantly (ferric chloride carotid) (67)
	YD-3	Small molecule	0.13 μM (PAR4-AP–induced human platelet aggregation) (69)	10 mg/kg (P.O.) impairs neointima formation in rats (76)
	ML354	Small molecule	140 nM (PAR4-AP–induced human platelet aggregation) (77)	Not reported
	BMS-986129	Small molecule	Not reported	Not reported

Abbreviations: AP, activating peptide; HUVECs, human umbilical vein endothelial cells; I.P., intraperitoneal; LLC, Lewis lung carcinoma; PAR, protease-activated receptor; P.O., per os.

In addition to these two clinically tested agents, several other small-molecule PAR1 antagonists continue to be used in experimental studies. The most commonly used are SCH79797 and SCH203009, which exhibit potent inhibition of PAR1-mediated events in multiple cell types (36, 37) and are effective in vivo (37–39). However, these agents have inferior efficacy and selectivity to atopaxar and vorapaxar, and there has been some concern regarding their PAR1-independent off-target effects (40, 41). Consequently, the two clinically tested agents, atopaxar and vorapaxar, have become the gold-standard small-molecule PAR1 antagonists for experimental studies. Despite the success in the development of small molecules, a remarkable array of distinct approaches to inhibit PAR1 function exists, as discussed below.

Pepducins Targeting PAR1

Pepducin-based PAR1 inhibitors continue to be investigated in detail, with one agent being evaluated in initial clinical studies. Pepducins are a unique class of GPCR antagonists purported to function by disrupting the interaction between the receptor and heterotrimeric G protein interface (**Figure 2**) (42). PAR1 was the first GPCR examined in initial proof-of-concept studies. Pepducins consist of a peptide sequence corresponding to a region in the intracellular loops (ICLs) or C terminus of the target GPCR that mediates G protein interaction. This short peptide sequence is conjugated to an N-terminal palmitate (pal) to facilitate membrane anchoring and alignment with the target G protein interaction site. Thus, pepducins appear to act by binding and sequestering G proteins competitively and thereby preventing downstream signaling induced by GPCR activation (42, 43). This mechanism of action implies the controversial contention that individual GPCRs couple to G proteins via unique determinants of interaction. However, despite concerns regarding specificity (44–46), the anti-PAR1 pepducin PZ-128 (previously P1pal-7) (47) was evaluated recently in a limited Phase I study in patients with coronary artery disease (**Table 2**) (48). PZ-128 targets the intracellular PAR1–G protein interface by mimicking ICL3 of PAR1 and has been shown to inhibit PAR1-mediated human platelet activation selectively (**Figure 2**) (49). In animal models, PZ-128 impaired arterial thrombosis rapidly and effectively in guinea pigs and baboons without any effect on bleeding (49). In the first human studies (48), PZ-128 was administered to 31 patients with clinical signs or risk factors of coronary artery disease and was shown to inhibit PAR1-mediated platelet activation selectively in a dose-dependent manner, and it did not perturb platelet activation by other agonists such as the PAR4 peptide agonist, adenosine diphosphate, or collagen. The plasma half-life of PZ-128 was 1.3–1.8 h, and these effects were reversible inside of 24 h. Intriguingly, a late spike in inhibitory activity at 6 to 24 h postinfusion was observed at certain doses and speculated to be due to redistribution of the highly lipophilic pepducin. In addition, notable acute allergic reactions were reported at the highest doses. Regardless, Phase II safety studies are planned for PZ-128 in approximately 600 patients undergoing nonurgent percutaneous coronary intervention (**https://clinicaltrials.gov/ct2/show/NCT02561000**).

Parmodulins: Small-Molecule Antagonists Targeting PAR1

Another distinct class of PAR1 antagonists are the very recently described parmodulins, a group of small molecules reported to exploit the promiscuous and functionally biased G protein coupling of PAR1 (50, 51). Similar to the pepducins, parmodulins bind to the intracellular face of PAR1 and interfere with activated receptor–G protein coupling (**Figure 2**) rather than the extracellular events of thrombin cleavage or ligand binding. In initial studies, a library of approximately 300,000 small molecules was screened for inhibitors of platelet granule secretion (51). These studies identified that compounds with a 1,3-diaminobenzene core inhibited PAR1-mediated platelet dense granule secretion selectively. Modification of the lead compound, ML161 (**Figure 3**), yielded a detailed structure-activity relationship set. These compounds were observed to target the cytoplasmic face of PAR1 without modifying the extracellular ligand-binding site of the receptor. Remarkably, they were observed to block PAR1 signaling via $G\alpha_q$ but not signaling via $G\alpha_{13}$ and were effective both in vitro and in vivo. A key predicted advantage of such discriminate inhibition of receptor–G protein coupling is the potential to modulate selectively the prothrombotic and proinflammatory effects of PAR1, which are predominantly $G\alpha_q$ mediated. Indeed, parmodulins were shown to inhibit prothrombotic PAR1 signaling without blocking the protective activated protein C (APC)-mediated PAR1 signaling pathways or inducing endothelial injury (**Table 2**) (51). These observations are in direct contrast to the effects of orthosteric PAR1 antagonists (e.g.,

atopaxar, vorapaxar, and other small molecules) that inhibit all PAR1-mediated signaling events indiscriminately. These findings raise the tantalizing possibility of functionally selective receptor antagonism that blocks pathological, but not cytoprotective, PAR1-mediated cell signaling events. However, issues remain regarding receptor selectivity: The first-in-class parmodulins appear to inhibit several GPCRs, including PAR4, that have commonality in helix 8 of the receptor, suggesting much further study is required to understand sufficiently the specificity and mechanism of action of this appealing class of PAR1 antagonists.

PAR2 AND PAR4 PHARMACOLOGY AND USE OF ANTAGONISTS

In addition to PAR1, PAR2 and PAR4 have emerged as important drug targets, whereas PAR3 has not been the target of any major drug development efforts so far. Similar to research on PAR1, work to develop agents that block irreversible proteolytic activation of PAR2 or PAR4 effectively has been met with enormous difficulty. Consequently, diverse strategies based on those taken with PAR1 have been pursued to develop agents that can inhibit PAR2 or PAR4 function. This has resulted in the development of blocking agents against PAR2 and PAR4 that appear effective in vitro and in vivo, including some small-molecule inhibitors that appear promising, but none have advanced to the clinic.

Development of Drugs Targeting PAR2

PAR2, the second member of the PAR family to be identified, is a trypsin-activated receptor. It is expressed broadly in multiple tissues and has been linked to inflammation, neurodevelopment, and cancer (52). Thus, blocking PAR2 activity may provide therapeutic benefit. However, PAR2 activation is also beneficial under certain conditions. Several studies using animal models are highly suggestive of important roles for PAR2 in arthritis (53), inflammatory bowel disease including colitis and radiation-induced intestinal inflammation (54, 55), and allergen-induced asthma (56). Similar to research on PAR1, diverse strategies have been taken to perturb PAR2 function, including the use of function-blocking antibodies, small-molecule inhibitors, and pepducins. The initial small-molecule antagonists developed for PAR2 were peptidomimetics based on the tethered ligand sequence generated by trypsin cleavage. ENMD-1068 (*N*1-3-methylbutryl-*N*4-6-aminohexanoyl-piperazine) inhibited trypsin-induced PAR2 activation and decreased joint inflammation in vivo (**Table 2**) (57); however, it failed to advance to clinical trials owing to a lack of efficacy (53). GB88 (5-isoxazoyl-Cha-Ilespiroindene-1,4-piperidine) (**Figure 3**) is a more recently developed, potent, reversible antagonist that blocks PAR2 activation by endogenous proteases and by the synthetic peptide agonist both in vitro and in vivo; it has been shown to attenuate inflammation in a rat model of colitis (58, 59). Interestingly, GB88 blocks only certain PAR2-stimulated signaling pathways selectively, including Ca^{2+} mobilization but not MAP kinase activation (**Table 2**). These findings indicate that blocking agents can function as biased antagonists.

PAR2 function-blocking antibodies generated against the tethered ligand sequence of PAR2 can inhibit trypsin-mediated cleavage in vitro and reduce PAR2-mediated joint inflammation in vivo but have not advanced to clinical trials (57). PAR2-targeted pepducins have been developed and shown to display both partial agonist and antagonist activity. The PAR2 pepducin P2pal-18S exhibits potent antagonist activity and can inhibit inflammatory responses in vivo (60). However, the PAR2 pepducin P2pal-21, which is homologous to the ICL3 of PAR2, exhibits partial agonist activity (42). Thus, similar to the case of PAR1, the utility of PAR2 pepducins as therapeutics for PAR2-mediated inflammatory conditions in vivo needs further investigation.

Development of Drugs Targeting PAR4

The development of pharmacological inhibitors of PAR4 has been significantly slower compared with agents targeting PAR1 and PAR2, due in large part to the limited knowledge of PAR4 physiological functions. Indeed, the most well-characterized role for PAR4 is in platelets, where it has long been thought to play a predominantly redundant role in thrombin-induced platelet activation, curbing enthusiasm for PAR4 inhibitor development. However, the clinical limitations of PAR1 inhibition observed in the vorapaxar clinical trials, coupled with recent studies uncovering additional PAR4 functions outside of platelets, have reignited interest in developing PAR4 antagonists, with some promising recent efforts.

PAR4 Function-Blocking Antibodies

The first PAR4 antagonist was a function-blocking rabbit polyclonal anti-PAR4 antibody. The antibody was raised against a peptide sequence corresponding to the thrombin cleavage site of human PAR4. The antibody inhibited thrombin cleavage of PAR4 in transfected Rat1 cells, abolished PAR4-mediated calcium signaling in mouse lung fibroblasts, and impaired thrombin-induced human platelet aggregation in the presence of concomitant PAR1 inhibition—albeit at very high concentrations (21). Despite this successful approach, only very recently have the function-blocking anti-PAR4 antibodies been used experimentally. Several recently developed function-blocking anti-PAR4 antibodies include similar rabbit polyclonal antibodies against the thrombin cleavage site of PAR4 (35) and those targeting the N-terminal anionic region of PAR4 (61), as well as a series of mouse monoclonal antibodies targeting both of these regions of the receptor (62). However, some of the anti-PAR4 antibodies display a surprisingly lack of specificity and inhibit not only thrombin-induced aggregation of human platelets but also platelet aggregation induced by agonists acting at PAR4, P2Y12, or GPVI receptors (61). Other PAR4 antibodies appear to have limited efficacy, as shown for a recently developed series of monoclonal anti-PAR4 antibodies that bind at or near either the anionic region or thrombin cleavage site of PAR4 and prevented thrombin cleavage and activation of PAR4 only partially in cell expression systems (62). In contrast, the recent polyclonal antibody targeting the thrombin cleavage site of PAR4 was highly specific and surprisingly efficacious when examined in similar platelet-based functional assays and in an ex vivo human thrombosis model (35). However, whether or not targeting PAR4 using a function-blocking antibody–based approach will be useful for future experimental and potential clinical studies remains to be determined.

Peptidomimetics of PAR4

As with early PAR1 antagonists, peptide- and peptidomimetic-based agents based on the tethered ligand sequence of PAR4 were similarly used as initial PAR4 antagonists. The key experimental reagent emerging from this approach was (*trans*-cinnamoyl)-YPGKF-NH_2 (tcY-NH_2) (63). This modified PAR4 agonist peptide was shown to bind to but not activate PAR4 and to abolish PAR4-induced aggregation of rat platelets at high concentrations (**Table 2**). tcY-NH_2 has also been reported to inhibit thrombin-induced aggregation of human platelets (64), although there is limited other evidence to indicate that tcY-NH_2 is an effective inhibitor of human PAR4. In addition, one report suggests tcY-NH_2 acts as an agonist at the PAR2 variant PAR2 F240S (65), raising serious concerns regarding its potential development as a therapeutic agent.

Pepducins Targeting PAR4

Anti-PAR4 pepducins were developed alongside the anti-PAR1 pepducins described above (42). In the initial study, the anti-PAR4 pepducin P4pal-10 (*N*-pal-SGRRYGHALR-NH_2) inhibited up to 85% of thrombin-induced aggregation of human and mouse platelets. In vivo assessment of P4pal-10 indicated impressive efficacy in a mouse tail bleeding time assay of platelet-dependent hemostasis (**Table 2**) (42, 43). However, as with the PAR1 pepducins, the specificity of P4pal-10 and other anti-PAR4 pepducins has been debated. For example, P4pal-10 displays activity against platelet activation induced by PAR1 (43), GPVI, and the thromboxane TP receptor (46). Indeed, P4pal-10 has been used as an inhibitor of global $G\alpha_q$ signaling in recent studies (66). In response to this broad-based $G\alpha_q$ inhibition, a distinct anti-PAR4 pepducin was developed that mimicked the first ICL of PAR4 [*N*-pal-ATGAPRLPST-NH_2 (P4-pal-i1)] rather than ICL3 of the receptor reported for PAR1 (67). In targeting a distinct intracellular region of the receptor, P4-pal-i1 appeared to function via a distinct mechanism that involved disruption of PAR1-PAR4 heterodimers and impairment of PAR1- and PAR4-mediated cellular events (**Table 2**) (67). Although clearly effective as an inhibitor of thrombin-mediated cellular effects, the lack of specificity of anti-PAR4 pepducins remains an important issue, and experimental studies using this approach should be interpreted with some caution.

PAR4 Small-Molecule Antagonists

Although several small-molecule PAR4 antagonists have been developed, little work has been published for any of these compounds. The indazole derivative YD-3 [1-benzyl-3(ethoxycarbonylphenyl)-indazole] is the most studied of this group (**Figure 3**) (68–70). YD-3 inhibited PAR4-induced aggregation of human platelets and partially inhibited human platelet aggregation in response to thrombin at relatively low concentrations (**Table 2**) (69, 71). These effects appeared to be specific, with no reported effect on aggregation induced by other platelet agonists. YD-3 has been used in a few studies to examine PAR4 function in the settings of platelet function (69, 71), angiogenesis (72–75), and inflammation (76). However, the in vivo utility of YD-3 is limited owing to its high lipophilicity, and ongoing efforts are aimed at optimizing the overall solubility and pharmacodynamic properties of the parent compound. ML354 (1-methyl-5-nitro-3-phenyl-1*H*-indole-2-methanol) is a recently discovered substituted indole with a molecular weight of 282 Da (**Figure 3**) and reasonable selectivity for PAR4 (77, 78). It inhibits PAR4-induced responses effectively in platelets (78). Finally, BMS-986120 is the lead compound from a series of imidazothiadiazole and imidazopyridazine derivatives that inhibit thrombin-induced aggregation of human platelets and was evaluated in Phase I clinical trials for safety and tolerability for the prevention, treatment, or both of thromboembolic disorders (**https://clinicaltrials.gov/ct2/show/NCT02208882**).

PAR VARIANTS OF BIOLOGICAL SIGNIFICANCE

Although researchers have reported numerous PAR single nucleotide polymorphisms (SNPs), only a few have been shown to affect PAR function and are important to consider for drug development. Interestingly, three potentially significant polymorphisms have been identified within PAR1, all of which are localized within the gene regulatory region. An adenine (A) to thymidine (T) transversion was identified in the intervening sequence, 14 nucleotides upstream from the exon 2 start site (IVS-14 A/T; rs168753). The A to T transversion variant affects PAR1 density and

function and has been shown to influence cardiovascular disease outcomes and cancer prognosis (79–81). Specifically, the AA genotype increases the risk of ischemic events in ST-elevation myocardial infarction patients (82) and is associated with an increased metastatic risk in renal carcinoma (83, 84). A second polymorphism occurs through a 13-base insertion/deletion that repeats the preceding sequence (5′-CGGCCGCGGGAAG-3′) at -506 within the promoter regulatory region (rs11267092) (85). The 13-base insertion/deletion variant has also been implicated in some clinical outcomes, with homozygosity of the insertion associated with a reduced risk of venous thromboembolism (85). Interestingly, the deletion allele is associated with improved prognosis in breast, stomach, and esophageal cancers (86–88). Finally, a cytosine (C) to T transition occurs 1,426 nucleotides upstream from the translation start site (-1426 C/T; rsXYZYXZYXZ). Studies indicate that the -1426 C/T variant is associated with recurrent pregnancy loss (possibly relating to lower levels of PAR1 in placental establishment) and preterm births (89, 90), suggesting an intriguing role for PAR1 function in pregnancy outcome.

In contrast to PAR1 variants, the known variants in PAR2 have not yet been correlated with significant clinical outcomes. Rather, the PAR2 variants appear to impact the pharmacology of the receptor. For example, the 240F > S polymorphism identified in PAR2 involves a region within the second extracellular loop of the receptor, and the S allele causes a significant reduction in sensitivity to both proteolytic and nonproteolytic receptor activation (65, 91). A second PAR2 variant involves a 621C > T polymorphism (rs631465) that appears to affect mRNA secondary structure and increase stability (92). The consequent increase in PAR2 levels has been linked to an increased risk of atopy in a cohort of Korean children (92), suggesting a role for PAR2 receptor levels in the regulation of inflammatory responses.

The most interesting receptor variants in the PAR family belong to PAR4, with very recent evidence suggesting SNPs in PAR4 regulate receptor expression and function in distinct patient populations (74, 93, 94). The 120A > T SNP (rs773902) of PAR4 is of particular note, with the 120T genotype associated with increased PAR4 expression and receptor sensitivity that may correlate to resistance to current antiplatelet drugs and poorer cardiovascular outcomes. The frequency of the 120T variant of PAR4 is remarkably high (>80% in some populations) and is racially dimorphic, occurring in 63% of self-identified blacks from a large North American cohort of 154 individuals compared with 19% of self-identified whites. Notably, the data from the Human Genome Diversity Project show SNP rs773902 is not region-specific, with 50–80% of people sampled in sub-Saharan Africa and about two-thirds of Papuans and Melanesians presenting with the 120T form of PAR4 (95). Remarkably, pharmacological studies showed that orthosteric PAR4 antagonism by the small-molecule antagonist YD-3 potently inhibited PAR4-induced platelet activation in patients genotyped as 120A but had no effect on platelets from patients genotyped as 120T (74), indicating this variant may have significant implications for drug development programs targeting PAR4.

PAR Biased Agonism and Drug Development

GPCRs are dynamic molecules that assume multiple different conformational states. Consequently, different agonists can stabilize unique active conformations of the same GPCR and facilitate activation of distinct signaling effectors such as heterotrimeric G proteins or β-arrestins. The activation of PARs occurs through proteolytic cleavage of the N terminus, revealing a tethered ligand sequence that binds intramolecularly to the receptor to trigger transmembrane signaling. This led to the assumption that different PAR-activating proteases would cleave at the same site to trigger similar signaling cascades and exhibit linear efficacy. However, this is not the case and is best exemplified by studies of PAR1.

In cultured human endothelial cells, thrombin activation of PAR1 is mediated by cleavage at arginine 41 (R41) and promotes coupling to $G\alpha_{12/13}$ and $G\alpha_q$ proteins. Thrombin-cleaved PAR1 then mediates activation of the small GTPase RhoA and other signaling effectors that promote disassembly of adherens junctions and reorganization of the actin cytoskeleton, resulting in disruption of the endothelial barrier, a hallmark of inflammation. Conversely, the anticoagulant protease APC, when bound to its cofactor endothelial protein C receptor, cleaves and activates endothelial PAR1 at a distinct site R46 in the N terminus (**Table 1**) (**Figure 4**) and induces endothelial barrier stabilization through activation of Rac1 and not RhoA signaling (96, 97). Unlike thrombin, APC activation of PAR1 does not promote coupling to $G\alpha_{12/13}$ or $G\alpha_q$ signaling but rather signals preferentially via β-arrestins to promote endothelial barrier maintenance (97). A synthetic peptide representing the APC-generated tethered ligand sequence recapitulates these responses (96). In addition, the compartmentalization of PAR1 in caveolar microdomains is required for APC cleavage and PAR1-mediated cytoprotective responses (98, 99), which is not a requirement for thrombin activation of PAR1. Collectively, these studies are among the first to illustrate that an endogenous GPCR exhibits biased agonism in response to activation by natural agonists (**Figure 4**).

APC is a naturally occurring anticoagulant and anti-inflammatory mediator that exists in human plasma. Human recombinant APC, also known as Drotrecogin alfa, was approved by the FDA for the treatment of severe sepsis, a life-threatening clinical condition resulting from uncontrolled inflammation and disseminated intravascular coagulation (100). However, APC was withdrawn from clinical use owing to a lack of efficacy and increased bleeding events (101). In addition to sepsis, APC has been shown to provide protection in multiple preclinical models of organ injury (102). APC cytoprotective activities include anti-inflammatory and antiapoptotic effects and stabilization of the endothelial barrier. Recent studies indicate that APC variants that signal selectively to cytoprotective activities and not to anticoagulant activities reduced mortality induced by endotoxin or bacteremia models of sepsis (103, 104). In addition, new APC variants that lacked anticoagulant activity and maintained signaling properties similar to wild-type APC reduced brain injuries caused by ischemic stroke and increased cardioprotection against ischemia/reperfusion injury in preclinical studies (102). These studies of APC illustrate clearly the opportunity and potential of exploiting PAR biased agonism for drug development.

Matrix metalloproteases (MMPs) have also been shown to cleave and activate PAR1 at distinct sites (**Table 1**) (105). MMP1 was first shown to cleave PAR1 at the proper site in breast carcinoma to elicit Ca^{2+} mobilization and breast cancer cell migration and invasion (106). MMP1 is expressed in vascular endothelial cells, platelets, fibroblasts, and macrophages. MMP1-activated PAR1 occurs through cleavage at D39 and promotes $G\alpha_{12/13}$-mediated RhoA signaling, MAP kinase activation, and platelet shape change but does not elicit robust Ca^{2+} mobilization or platelet aggregation, in contrast to thrombin (107, 108). A peptide agonist that mimics the MMP1-generated PAR1 tethered ligand caused comparable effects. Thrombin and MMP1 also induced distinct responses in vascular smooth muscle cells and arterial stenosis following arterial injury (105). In addition to MMP1, MMP13 can cleave and activate PAR1 at a distinct F43 site in ventricular myocytes and may promote biased signaling, but this remains to be explored fully (109). Inflammatory neutrophils secrete elastase and proteinase-3, which can cleave and activate PAR1 through distinct sites—L45 and A36, respectively (**Table 1**). In contrast to thrombin-activated PAR1, elastase and proteinase-3 activated PAR1 signals preferentially through $G\alpha_i$ to promote MAP kinase activation (110). Synthetic peptides that represent the elastase- or proteinase-3–generated tethered ligand sequence reproduced similar phenotypes in cell models expressing PAR1 exogenously. However, the physiological context in which elastase or proteinase-3 may function to activate PAR1 selectively to promote biased signaling remains to be determined.

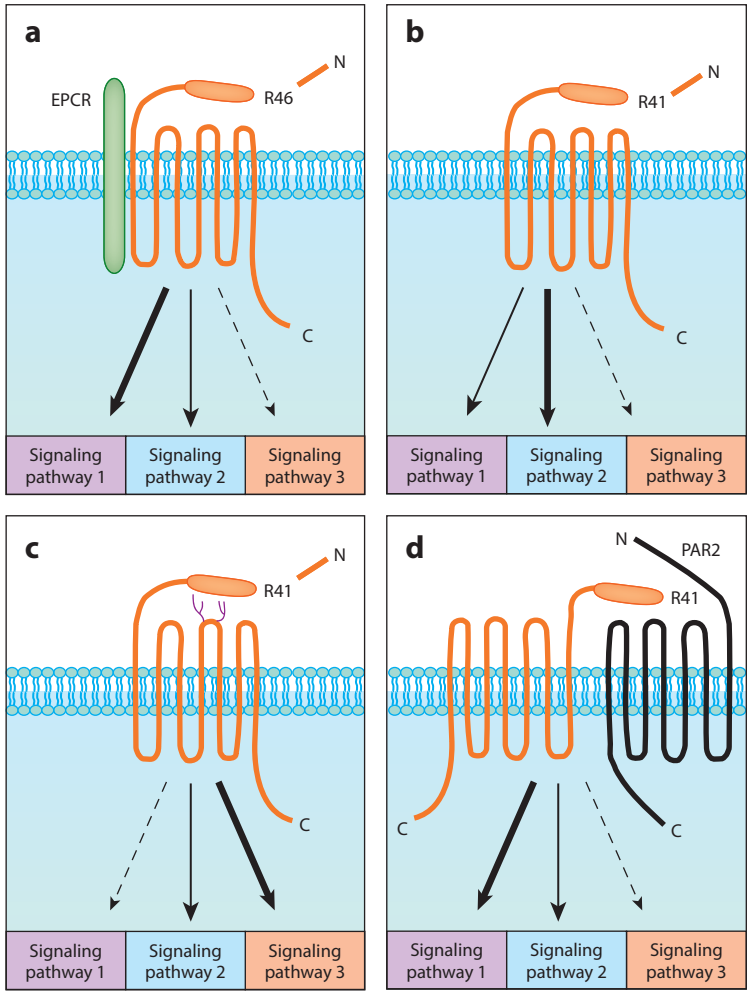

Figure 4

PAR1 biased signaling is induced by specific proteases, glycosylation, and dimerization. (*a*) Activated protein C (APC) bound to its cofactor, endothelial protein C receptor (EPCR), can bind to and cleave PAR1 at arginine 46 (R46), generating a distinct tethered ligand that activates the receptor, which signals selectively to a cytoprotective and anti-inflammatory pathway in endothelial cells. (*b*) In contrast, thrombin binds to and cleaves PAR1 at R41, resulting in rapid activation of signaling responses that promote vascular inflammation. (*c*) PAR1 is glycosylated extensively at asparagine residues (*purple*) localized to extracellular loop 2 and influences tethered ligand–receptor interactions and G protein coupling specificity. (*d*) PAR1-PAR2 dimers exist in different cell types, including endothelial cells, and cleavage of PAR1 by thrombin can generate a tethered ligand sequence that can bind intramolecularly to an adjacent PAR2 to promote transmembrane signaling. Arrow weight indicates signaling strength in all panels.

PAR2 and PAR3 can also be cleaved and activated by multiple extracellular proteases (**Table 1**) and appear to promote biased signaling responses. However, further studies are needed to better understand the selectivity, receptor-specific signaling properties, and physiological significance of protease-specific biased signaling before considering the potential for drug development. In addition, many proteases, such as MMPs and neutrophil proteases, target a broad variety of

proteins, including other PARs, as well as PAR-independent cellular functions in the vasculature and other tissues. Thus, strategies to manipulate a specific protease function to activate a given PAR selectively or inhibit protease-mediated PAR1 activation utilizing specific protease-selective inhibitors may be challenging.

Posttranslational Modification of PARs

Posttranslational modifications are integral to the function of GPCRs. Besides phosphorylation, most if not all Class A GPCRs are modified by asparagine (N)-linked glycosylation. The majority (approximately 90%) of Class A GPCRs contain N-linked glycosylation N-X-S/T consensus sites within their N terminus, whereas about 30% of receptors contain consensus sites within their extracellular loops (111). However, the extent and full use of consensus sites likely varies with a given GPCR and is probably different in normal versus pathological conditions. Given that glycosylation occurs on the extracellular domains of GPCRs, sites that are also important for ligand recognition, it is not surprising that glycosylation can influence GPCR-ligand interactions and thus is an important consideration for drug discovery.

PAR1 contains five consensus sites for N-linked glycosylation, three in the N terminus and two in extracellular loop 2, and all appear to be modified by glycosylation (112). PAR2 contains an N-linked glycosylation site in the N terminus and one in extracellular loop 2 (113). One N-linked glycosylation consensus site exists in the N terminus of PAR4, whereas PAR3 has two sites, one in the N terminus and one in extracellular loop 3. Consistent with the known function for N-linked glycosylation of GPCRs in proper folding of the nascent proteins during translation and export to the cell surface, glycosylation of PAR1 at the N terminus is important for efficient transport to the cell surface. However, glycosylation of PAR1 at extracellular loop 2 serves a distinct function. Recent work showed that PAR1 glycosylation at extracellular loop 2 stabilizes a distinct thrombin-induced active conformation of PAR1 that couples preferentially to $G\alpha_{12/13}$ over $G\alpha_q$ protein but has no influence on receptor coupling to $G\alpha_i$ or β-arrestin-1 (**Figure 4**) (114). The heterogeneity of glycosylation indicates that GPCRs may exist as populations of receptors containing distinct glycan structures. This suggests that PAR1 may exist as an ensemble of active states that uses different molecular determinants to couple to distinct G protein subtypes or β-arrestins. In addition to PAR1, glycosylation of PAR2 at the N terminus was shown to affect tryptase but not trypsin cleavage and activation of the receptor (113), indicating that glycosylation affects protease recognition and receptor activation directly. Naturally occurring mutations in the N-linked glycosylation consensus sequences of the rhodopsin GPCR have been linked to retinitis pigmentosa (115, 116). To our knowledge, naturally occurring mutations have yet to be identified in any PAR, and changes in the status of PAR glycosylation in specific disease states have not been explored fully.

The ability of different proteases to cleave PARs at distinct sites results in the generation of unique tethered ligands and active conformations that will likely influence both ligand-induced phosphorylation and ubiquitination. Phosphorylation of GPCRs is important for β-arrestin recruitment. β-Arrestins are multifunctional adaptor proteins that facilitate GPCR uncoupling from G protein signaling and internalization, a process important for signal termination. In addition, β-arrestins act as scaffolds that promote signaling through various MAP kinase cascades (117). Previous studies showed that the extent and sites of phosphorylation that occur on a particular GPCR in response to ligand activation not only affect the stability of β-arrestin–GPCR interaction but also direct β-arrestin activity toward specific functions. This phenomenon has been termed the barcode hypothesis. This hypothesis is best illustrated by the β-adrenergic receptor, which is phosphorylated differentially in response to activation with different ligands, and the M3

Table 3 PAR posttranslational modifications

Receptor	Ligand	Posttranslational modification	Cellular function
PAR1	Thrombin SFLLRN	Phosphorylation	Desensitization, internalization, degradation (141, 142)
	Thrombin SFLLRN	Ubiquitination	Internalization, p38 MAP kinase activation (13, 143)
	NA	Glycosylation	Biosynthesis, G protein coupling specificity (112, 114)
	NA	Palmitoylation	Internalization, adaptor protein recognition (121)
PAR2	SLIGKV	Phosphorylation	Desensitization, β-arrestin binding (12, 144)
	SLIGKV	Ubiquitination	Lysosomal degradation (124)
	NA	Glycosylation	Agonist sensitivity (113)
	NA	Palmitoylation	Agonist sensitivity, desensitization, internalization (122, 123)
PAR3	NA	Not known	NA
PAR4	AYPGKF	Phosphorylation	Not detected (145)
	NA	Not known	NA

Abbreviations: MAP, mitogen-activated protein; NA, not applicable; PAR, protease-activated receptor.

muscarinic receptor, which is phosphorylated differentially by the same ligand when expressed in different tissues. Both result in distinct β-arrestin functions (118, 119). Although PAR1 and PAR2 are phosphorylated robustly following activation (**Table 3**) (12, 120), no studies have examined differential phosphorylation of any PAR in response to activation with different proteases and the impact on cellular behavior.

Similar to phosphorylation, many GPCRs including PAR1 and PAR2 are posttranslationally modified by palmitoylation and ubiquitination (**Table 3**) (13, 121–124). Palmitoylation occurs through the covalent attachment of pal, a 16-carbon saturated fatty acid, to cysteine residues via a thiester linkage. PAR1 and PAR2 are palmitoylated on juxtamembrane C-tail cysteine like other GPCR residues that appear to facilitate palmitoyl group insertion into the lipid bilayer. Palmitoylation of PAR2 regulates receptor expression, agonist sensitivity, desensitization, and internalization (122, 123). In contrast, palmitoylation of PAR1 is important for proper adaptor protein recognition of tyrosine-based sorting motifs and consequently is critical for retaining appropriate amounts of receptor at the cell surface required for appropriate thrombin responses (121). In addition to trafficking, PAR1 palmitoylation modulates receptor–G protein coupling, a phenotype observed with a PAR1 cysteine mutant (125). A small-molecule inhibitor, JF5, blocked PAR1 activation of $G\alpha_q$ signaling and required the putative eighth helix formed by palmitoylation (50). However, this JF5 inhibitor effect was not specific to PAR1, as signaling by the chemokine CCR4 and serotonin 5-HT_{2A} GPCRs containing cysteine residues and a putative eighth helix were similarly inhibited. These data suggest that palmitoylation of certain GPCRs appears to generate an intracytosolic interface that can be targeted for therapeutic development.

Ubiquitin is a small, 76–amino acid protein that is recognized by proteins that harbor ubiquitin-binding domains. The major function of ubiquitin is to serve as a signal for sorting of activated GPCRs from endosomes to lysosomes for degradation. This is clearly the case for agonist-activated PAR2 (124). However, new work suggests that ubiquitination of certain GPCRs functions to recruit adaptor proteins that promote signaling responses. This has been demonstrated recently for thrombin-activated PAR1. Upon activation with thrombin, PAR1 is ubiquitinated rapidly via K63-linked ubiquitin, which promotes binding of TAB2 via a ubiquitin-binding Npl4 zinc finger domain. TAB1 is then recruited to activated PAR1-TAB2 and induces p38 MAP kinase

autophosphorylation and activation to promote endothelial barrier disruption (13). The ubiquitination of the purigenic P2Y1 receptor functions similarly to promote p38 MAP kinase activation in endothelial cells (13), but whether other PAR1 activating proteases cause an analogous response is not known. Thus, it is important to consider the type of posttranslational modifications that occur on a given PAR in response to different activating proteases in order to define the signaling responses that drive a particular physiological process, which has important implications for therapeutic development.

PAR Dimerization and Drug Development

PAR dimerization has important implications for drug development, as most drugs are being developed to target PAR monomers. Thus, researchers must consider how pharmacological perturbation of PAR monomers will affect receptors that exist as dimers or higher-order oligomers. Here we discuss studies that show that PARs interact with each other to form dimers and possibly higher-order oligomers in normal cells and disease states.

Most cell types express more than one PAR, and the activity of these PARs can be modulated by interaction with each other. This is best exemplified in studies of endogenous PARs expressed in the cells of the vasculature. In human platelets, PAR1 is coexpressed with PAR4, whereas PAR3 and PAR4 are coexpressed in mouse platelets. Thrombin can bind to and cleave mouse PAR3; however, cleaved PAR3 does not appear to signal autonomously but rather functions as a cofactor to facilitate efficient cleavage and activation of PAR4 (126). Similarly, PAR1 appears to enhance the cleavage and activation of PAR4 in human platelets. PAR1 and PAR4 have also been shown to form a complex in human platelets (67), and this contributes to efficient activation of platelets by thrombin. In contrast to PAR3, activated PAR4 couples efficiently to G proteins and appears to function by promoting sustained signaling and late phases of platelet aggregation (127, 128). The ability of a PAR to cofactor other PARs would necessitate that the two receptors be in close proximity, likely in the form of a heterodimer.

In endothelial cells, PAR1 and PAR3 are both expressed, and loss of PAR3 attenuates the ability of thrombin to promote endothelial barrier disruption (129). This response has been attributed to the ability of PAR3 to modulate PAR1 preferential signaling to $G\alpha_{13}$. APC cleaves PAR1 as well as PAR3 and promotes cytoprotective signaling in endothelial cells, neurons, and mouse podocytes (130–132). Moreover, APC induces PAR1-PAR3 heterodimer formation, but it is not clear how dimer formation contributes to induction of PAR1- versus PAR3-specific signaling responses in distinct cell types.

PAR2 is typically expressed at low levels in endothelial cells and is increased during inflammation (133). Under conditions with elevated PAR2, thrombin cleavage of the PAR1 N terminus unmasks a tethered ligand domain that can bind in *trans* to activate PAR2 via an intermolecular mechanism, which elicits distinct signaling responses compared with either receptor protomer alone (**Figure 4**). The initial studies with endothelial cells used PAR1-blocking antibodies and cross-desensitization experiments to show activation of PAR2 occurred via thrombin cleavage of PAR1 (23). In more recent work, PAR1 was shown to transactivate PAR2 during sepsis progression (134). Under these pathological conditions, PAR1 signaling switched from vascular disruptive to vascular protective as a consequence of increased endothelial PAR2 expression. Consistent with these studies, in cytokine-treated endothelial cells, expression of PAR2 enhanced the capacity of thrombin-activated PAR1 to signal differentially compared to PAR1 alone (135). Coimmunoprecipitation, fluorescence resonance energy transfer, and bioluminescence resonance energy transfer studies provide substantial evidence that PAR1 and PAR2 form a dimer (136). Moreover, the thrombin-activated PAR1-PAR2 dimer signals via β-arrestins and Rac1, rather than G proteins

and RhoA signaling mediated by the receptor protomer. These studies suggest that in addition to different proteases that cleave at distinct sites in the PAR1 N terminus, other receptor properties can influence signaling, including compartmentalization into plasma membrane microdomains, posttranslational modifications, and dimerization.

CONCLUSIONS

Although researchers have made considerable progress in understanding PAR function in vivo as well as in delineating receptor biased signaling properties both in vivo and in vitro, the FDA has approved only one drug targeting the PAR1 orthosteric binding site. Thus, unprecedented opportunities exist to develop new pharmacological agents targeting PARs, including orthosteric and allosteric modulators. Both orthosteric and allosteric ligands can be developed to interact with a specific GPCR in a manner that can promote biased signaling through coupling to distinct signaling effectors. Thus, major efforts are needed to design small molecules in a manner that promotes PAR biased signaling, which may result in greater clinical efficacy and a reduction in adverse side effects. Indeed, the PAR2 small-molecule inhibitor GB88 and the PAR1 parmodulin ML161 inhibitor block some but not all receptor-mediated signaling pathways and appear promising. In addition, structure-based drug design is an attractive and plausible approach for developing a new generation of PAR inhibitors and is feasible, given the recent determination of the high-resolution crystal structure of PAR1 bound to vorapaxar (137). Thus, rapid developments in GPCR crystallography approaches will likely result in other PAR structures in the near future, which can complement other established, high-throughput screening approaches. Together, these diverse strategies will help researchers develop novel PAR pharmacological entities that may be more selective and effective with fewer adverse side effects.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

The authors thank Drs. Shauna French and Michael Dores for assistance in researching and preparing this work.

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