Protease activated receptor 2 (PAR2) modulators: a patent review (2010-2015)

Mei-Kwan Yau, Junxian Lim, Ligong Liu, David P. Fairlie*

Division of Chemistry and Structural Biology, Institute for Molecular Bioscience, The University of Queensland, Brisbane, Queensland 4072, Australia

* Corresponding author: Phone: +61-733462989. Fax: +61-733462990. Email: d.fairlie@uq.edu.au

Abstract

Introduction: Protease activated receptor 2 (PAR2) is self-activated upon cleavage of its N-terminus by a proteolytic enzyme, especially a serine protease. PAR2 has been implicated in several diseases, including inflammatory, cardiovascular, gastrointestinal, respiratory and metabolic diseases as well as in metastatic cancer, pain and neurodegenerative conditions, making it an important prospective drug target. There are no known endogenous ligands for PAR2, so the availability of potent exogenous agonists and antagonists can be helpful for studying physiological functions of PAR2, determining its importance in diseases, and as leads to new pharmaceuticals.

Areas covered: This review covers agonist-, antagonist-, antibody- and pepducin-based modulators of PAR2 reported in patent applications between 2010–2015, along with their available structure-activity relationships, biological activities and potential uses for studying PAR2.

Expert opinion: In the last six years, substantial efforts have been made towards developing PAR2 modulators, but most still lack potency or selectivity or have poor pharmacokinetic profiles. The majority of the reported PAR2 modulators were assessed by measuring Gαq protein-mediated calcium release in cells. This may be insufficient to fully characterize ligand function, since emerging evidence has indicated that different ligands signal through PAR2 to different G-protein dependent and independent signaling pathways. PAR2 pathophysiology seems linked to specific signaling cascades that are differentially affected by different proteases or small

molecule agonists or antagonists. Therefore, it may be feasible to develop biased ligands as drugs that can selectively modulate one or more specific signaling pathways linking PAR2 to a specific diseased state or physiological process, without limiting beneficial or protective PAR2 signaling. Accordingly, potent, orally bioavailable, pathway- and receptor-selective PAR2 modulators may be an achievable goal to realizing effective drugs that can treat PAR2-mediated diseases.

Keywords

Protease activated receptor 2 (PAR2), agonist, antagonist, antibodies, patent.

1. Introduction

Protease activated receptors (PARs) belong to a unique family of G protein-coupled receptors (GPCRs) whose members are not activated by an endogenous ligand. Instead, the N-terminus of PARs is cleaved by proteases at a specific site and the newly formed N-terminus, known as tethered ligand (TL), then binds elsewhere on the receptor to initiate receptor activation [1-3]. There are four subtypes of PARs, PAR1, 2, 3 and 4, but only PAR2 is not activated by thrombin. The structure of PARs is similar to other GPCRs, consisting of an extracellular N-terminus, an intracellular C-terminus, seven transmembrane helices connected by three extracellular and three intracellular loops, and a small helix within the intracellular C-terminus. PAR1 and PAR3, but not PAR2, contain a negatively charged hirudin-like region on the N-terminus that binds to the exosite I of thrombin. PAR4, which does not contain a hirudin-like region, interacts with the active site of thrombin [4].

The N-terminus of PAR2 can be cleaved by proteases, such as trypsin, tryptase, factor Xa/VIIa, matriptase, granzyme A, kallikreins (KLK 2/4/5/6/14), MMP-1, cathepsin S, elastase, acrosin, HAT, TMPRSS2, chitinase, bacterial gingipains, Der P1–3, Pen C 13 and testisin to initiate PAR2 activation [2, 5]. These enzymes are not considered to be endogenous agonists for PAR2 since they are not bound directly to the activated receptor. No endogenous agonists have been reported yet for PAR2. PAR2 is primarily cleaved at the canonical site ($R^{36}S^{37}$) by serine proteases, such as by trypsin, tryptase, factor VIIa, matriptase and others [5]. However, it also can be cleaved by other proteases (e.g. elastase, cathepsin S) at a different site, either inactivating ('disarming') PAR2 or producing different TLs [2, 6-9] that initiate different signaling profiles, resulting in the phenomenon of biased agonism [10, 11]. PAR2 couples to G proteins $G\alpha_q$, $G\alpha_i$, $G\alpha_s$, $G\alpha_{12/13}$ and also β-arrestins 1 and 2 to induce downstream signaling [12]. $G\alpha_q$ activates production of inositol triphosphate and diacylglycerol, resulting in downstream activation of protein kinase C and mobilization of calcium ions [13]. The $G\alpha_i$ pathway predominantly inhibits cAMP via inhibition of adenylyl cyclase while also activating the mitogen-activated protein kinase (MAPK) pathways. In contrast, the $G\alpha_s$ pathway activates adenylyl cyclase, which stimulates cAMP production. The $G\alpha_{12/13}$ pathway activates the c-Jun N-terminus kinases (JNK) and RhoA leading to migration, differentiation and growth [14]. Activation of distinct signaling pathways via G proteins or β-arrestins can be associated with different diseased states [10, 15]. Activation of PAR2 by agonists or proteases has been shown to trigger multiple signaling pathways such as intracellular calcium (iCa^{2+}), MAPK and phosphorylation of extracellular signal-regulated kinases (ERK1/2), cAMP, Rho kinase, and nuclear factor κB (NF-κB) pathways [15-17].

PAR2 is expressed on numerous epithelial and endothelial cells, including of the lung, gastrointestinal tract, skin, kidney, pancreas, liver, heart, smooth muscle cells, fibroblasts, nerves and immune and inflammatory cells (T-cells, monocytes, macrophages, neutrophils, mast cells and eosinophils) [18-21]. Therefore, it is not surprising that PAR2 has roles in physiological processes associated with respiratory and gastrointestinal functions, tissue metabolism, immunity and neuronal signaling. Monocytes are known to express functional PAR2 and activation of the receptor led to the secretion of inflammatory cytokines IL6, IL8 and IL1β [22, 23]. Activation of PAR2 on macrophages up-regulates CCL2 and expression of other cytokines [22, 24]. PAR2 is widely expressed on astrocytes, microglia and neurons, and studies have shown PAR2 to be implicated in neuroinflammation and neurodegeneration [25, 26]. On the other hand, PAR2 has been reported to be neuroprotective in ischemic injury, infection and Alzheimer's disease [27-29]. PAR2 is also expressed throughout the gastrointestinal tract and mediates salivary, gastric and pancreatic exocrine secretion [30]. PAR2 stimulation has been reported to be protective in certain models of acute pancreatitis, with PAR2 deficient mice exhibiting more severe pancreatic oedema, acinar cell injury and pancreatic inflammation [31-33]. Evidence from PAR2 gene deletion in mice, PAR2 expression, PAR2 activation and in some cases PAR2 antibodies, links PAR2 to either detrimental or beneficial effects in different tissues or different disease settings.

Since the discovery of PAR2, a great deal of basic research has been conducted towards developing potent PAR2-selective ligands. In the last few years, researchers have become more focused on understanding biased pathway- and receptor-selective ligands for PARs. This review focuses on PAR2 ligands, antibodies and pepducins

reported in patent applications between 2010–2015 and their potential therapeutic uses related to PAR2 modulation.

2. PAR2 signaling and disease

Activation of PAR2 by proteases or a synthetic agonist is thought to involve formation of multiple, as yet undefined, distinct receptor conformations that can elicit unique downstream signaling events. These signaling events can be canonical G protein-mediated responses such as $G\alpha_q$-mediated calcium mobilization or G protein-independent β-arrestin-ERK1/2 phosphorylation. Studies have identified certain PAR2 ligands as being biased, being functionally selective through specific signaling pathways [2, 10]. Our previous work has demonstrated a PAR2 ligand that selectively inhibits $G\alpha_q$-calcium-PKC signaling and *in vivo* inflammatory responses, while acting as an agonist in activating ERK and Rho pathways [15]. PAR2 activation and upregulation has been associated with a number of pathophysiological conditions, such as metabolic dysfunction, arthritis, multiple sclerosis and cancers (Table 1). PAR2 also plays important roles in modulating inflammatory [19, 34, 35], gastrointestinal [36, 37], cardiovascular [38, 39], respiratory [40-42], and metabolic diseases [43-45], asthma [41, 46] and cancers [47-49].

However, in certain settings and diseased states, PAR2 activation exerts protective effects. Activation of PAR2 has been shown to prevent histamine-induced bronchoconstriction [50], ischemia/reperfusion injury [51], colitis [52] and to be important in wound healing [53, 54]. Thus, PAR2 may be protective and beneficial, or

damaging and detrimental, depending upon the tissue type, the PAR2 ligands used and their distribution properties, and the presence of other environmental stimuli.

3. PAR2 peptidic and small molecule agonists

Synthetic PAR2 agonist peptides mimicking the canonical TL sequences (SLIGRL, rodent; SLIGKV, human) and ending with a C-terminal amide have been used heavily to study the pathophysiology of PAR2. The C-terminal amide is more important for agonist potency compared to the N-terminal amine. The rodent hexapeptide (SLIGRL-NH_2) was 2-fold more potent than the human analogue (SLIGKV-NH_2) in most cell-based assays [75-77]. However, these six-residue peptides are not very potent, high concentrations of these peptides were needed to cause agonist effects. Structure-activity relationship (SAR) studies revealed that the agonist activity of SLIGRL-NH_2 was improved by 10–20 fold when the N-terminal serine (position 1) was replaced by a 2-furoyl group (abbreviated as 2f) [78] or other heterocycles [79]. The adjacent leucine residue at position 2 was crucial for PAR2 selectivity. Replacing it with phenylalanine, 4-fluorophenylalanine in particular, resulted in PAR1 activation [77]. Position 3 favored isoleucine or other groups of similar size. The fourth residue, glycine, could be replaced with other amino acids without significant loss in potency. Positively charged residues were preferred at the fifth position, but hydrophobic amino acids were also tolerated. The sixth residue, leucine, was the least important, as substitution by many other amino acids did not show significant change in agonist potency [77, 79]. However agonist potency was improved when leucine was substituted with tyrosine, 4-nitrophenylalanine or 3,4-dichlorophenylalanine [79].

One of the most commonly used PAR2 peptide agonists is 2f-LIGRLO-NH_2. This peptide activated PAR2 with EC_{50} 0.2 μM in a calcium mobilization assay on HT29 cells and similar effects vs. several other cell types [78, 80]. Peptides tend to be proteolytically cleaved by proteases, making them highly unstable *in vivo* and they have low oral bioavailability in part due to their poor membrane permeation and rapid clearance from blood. A patent from Peptron Inc reported 2f-LIGRLO-NH_2 derivatives with *N*-alkyl amino acids incorporated improved agonist potency and proteolytic stability of PAR2 peptides [81]. It is known that *N*-alkyl amino acids on peptides improve biological stability and lipophilicity [82]. The most potent PAR2 agonist reported in this patent was peptoid **2** containing an isopropyl side chain on the amide nitrogen (Figure 1). Peptoid **2** (EC_{50} 42 nM) was ~3-fold more potent than **1** (EC_{50} 120 nM) when assessed in an intracellular calcium mobilization assay on HCT-15 human colorectal adenocarcinoma cells. It also displayed longer plasma stability than **1**, remaining intact in plasma over 48 h whereas peptide **1** was completely degraded. There are other PAR2 agonists (**3–6**, Figure 1) [80, 83-86] that are not reported in patents and are therefore not discussed in this review. AY77 (**5**) was reported in a patent [87] and it is a small agonist that activated PAR2-induced calcium release with EC_{50} 33 nM in human PAR2 (hPAR2) transfected Chinese hamster ovary (CHO) cells [86]. Synthetic agonists are particularly useful for studying the functions of PAR2, since no endogenous agonist is yet known for this receptor, and may relieve airways inflammation according to reports [40, 41, 46].

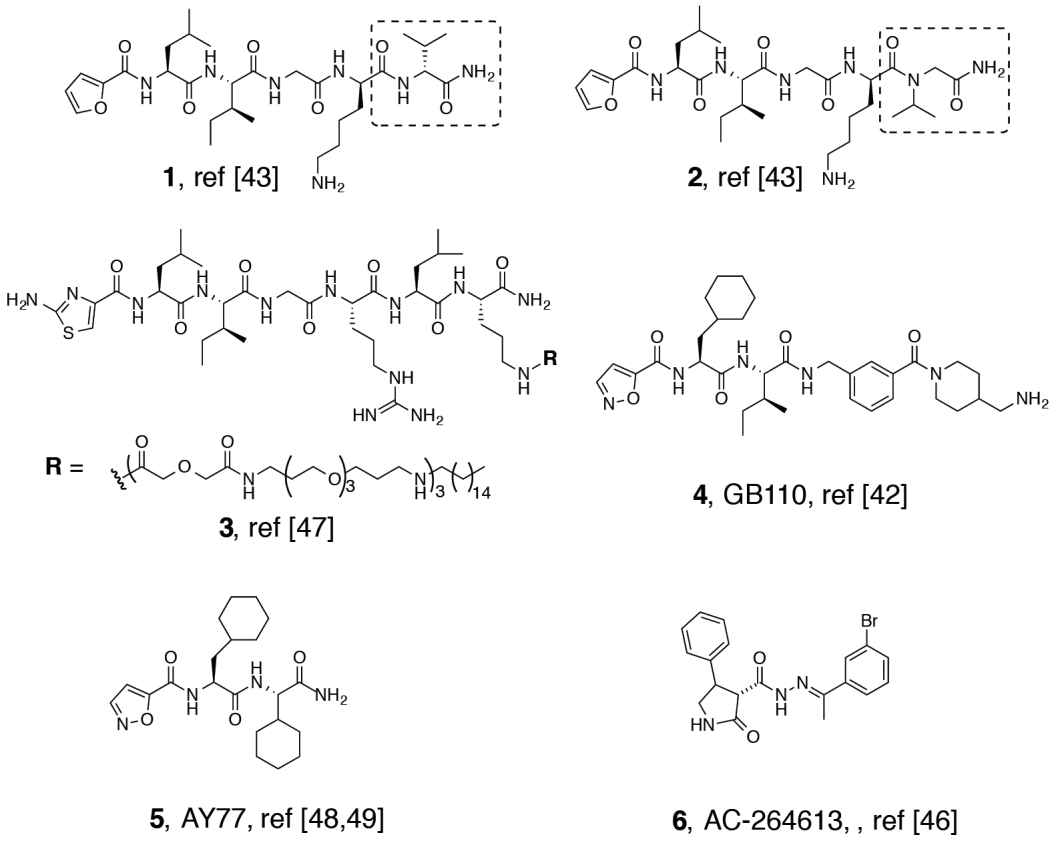

Figure 1. PAR2 peptidic and non-peptidic agonists **1**–**6**.

4. PAR2 antagonists

The functional properties of PAR2 activation have been investigated extensively by PAR2 KO studies (Table 1), indicating that inhibition of PAR2-mediated functions could have potential therapeutic applications in multiple human diseases, including arthritis, obesity, cardiovascular diseases, brain disorders, cancers and other inflammatory diseases such as colitis, inflammatory bowel disease (IBD), dermatitis and pancreatitis [2]. Therefore, potent and selective PAR2 antagonists could be potential drugs to treat these or related conditions. Antagonists of PAR2 reported to date include peptides, small molecules, antibodies and pepducins. In this review, the biological properties of PAR2 antagonists, mainly small molecules, as reported in patent applications (2010–2015) are briefly summarized.

4.1 Peptide antagonists

The first reported PAR2 peptide antagonists were FSLLRY-NH_2 and LSIGRL-NH_2, which inhibited (IC_{50} 50–200 μM) trypsin-induced calcium release in KNRK-PAR2 cells [88]. Addition of two charged residues (arginine) at the N-terminus of FSLLRY-NH_2, to give RRFSLLRY-NH_2, was reported to slightly improve antagonist potency [89]. The patent reporting this peptide showed that it had skin-lightening effects and might be incorporated into skin care products or used to treat inflammatory skin diseases [89].

Another group of PAR2 peptide antagonists was reported for the possible treatment of skin diseases, as well as pain. These peptides contained six L-amino acid residues capped by an acetyl group at the N-terminus and an amide at the C-terminus. They showed 50–90% inhibition of calcium release induced by 2f-LIGRL-NH_2 at 1mg/mL in human keratinocytes. For example, one of the peptide antagonists Ac-FFWFHV-NH_2 reported in this patent inhibited calcium release and also reduced the release of calcitonin gene-related peptide (CGRP) by 80% at 0.5 mg/mL. The PAR2-mediated release of CGRP was induced by sensitization of TRPV1. At the same peptide concentration (0.5 mg/mL), Ac-FFWFHV-NH_2 also inhibited IL-6 production. Moreover, this peptide induced proliferation of human keratinocytes by 23% at 25 μg/mL, suggesting that it might be able to improve the barrier function of skin. Additionally, it promoted healing of human keratinocytes by 45% at 0.5 mg/mL and it was photo-protective in human dermal fibroblasts at 0.01 μg/mL. This peptide also

improved skin hydration *in vivo* and reduced IL-8 production when induced by cosmetic allergens, such as cinnamal and farnesol [90].

Synthetic peptides mimicking the PAR2 cytoplasmic C-terminus have been reported to have a possible application in treating cancer [91]. Upon PAR2 activation, the C-terminus of PAR2 binds to downstream signaling pleckstrin-homology (PH) domain containing proteins such as Etk/Bmx. The C-terminus sequence of PAR2 that interacts with the PH domain is SHDFRDHA. Therefore, peptides containing this sequence (minimum 8 amino acids in length) can interfere the interaction of the PAR2 C-terminus with PH-domain containing proteins and thus inhibit the associated signaling events. These peptides were able to penetrate the cell membrane and were suggested to be beneficial for cancer treatment [91].

4.2 Small molecule antagonists

In the past few years, there has been growing interest in developing small molecule antagonists for PAR2, since they are usually more stable *in vivo* than peptides and possess better pharmacokinetic profiles including greater membrane permeability and oral bioavailability. Only a few small molecule PAR2 antagonists were reported before 2016 in scientific journals (**7–15**, Figure 2), but most only inhibit PAR2-induced functions at micromolar/millimolar concentrations when applied to various cell lines (e.g. HT29, NCTC2544-PAR2, A549) [80, 92-98]. Some of these antagonists also showed biased signaling by selectively inhibiting one signaling pathway but not others [15, 96, 99].

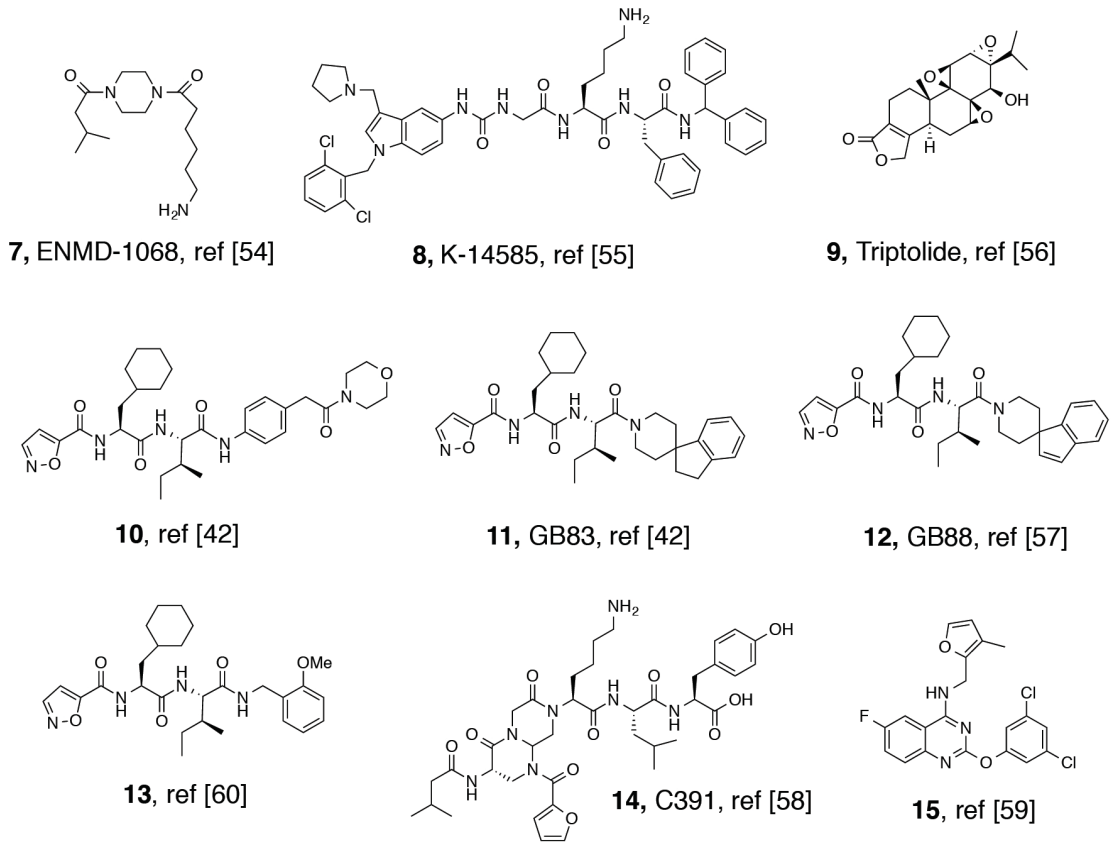

Figure 2. PAR2 antagonists reported in scientific journals.

Multiple patents published between 2010–2015 have reported small molecule PAR2 antagonists, with molecular weights (MW) ranging between 200–600 Da (Table 2). They were mainly examined *in vitro* for inhibition of calcium release induced by known PAR2 agonists in various cultured human cell types, but few have been shown to act specifically on PAR2 or to inhibit key protease, peptide and nonpeptide agonists of PAR2. Some of these antagonist compounds were shown to elicit responses in rodent models of disease. It is important to point out, however, that not all disease models are associated specifically with PAR2 activation, thus *in vivo* effects are not necessarily linked to PAR2 modulation. This should be taken into account when interpreting *in vitro* and *in vivo* data. All PAR2 antagonists listed in Table 2 will be discussed in detail later.

4.2.1 Piperazine and piperidine derivatives

Some piperazine derivatives similar to **7** (ENMD-1068) were reported by NeoPharm in 2010, with **16** (Figure 3) being the most potent compound reported in the patent. This compound (2.5 mM) showed complete inhibition of calcium release induced by SLIGKV (2.5 μM) in HCT-15 colon carcinoma cells, a PAR2-overexpressing cell line, and was claimed to be useful for treating atopic dermatitis [100]. Compounds **7** and **16** lacked potency and are likely to have off-target effects at the millimolar concentrations required for efficacy.

In 2012, a number of substituted piperidine/piperazine urea derivatives (Figure 3) were reported as low micromolar PAR2 antagonists for the inhibition of calcium release against trypsin (EC_{50} concentration) in human PAR2 transfected 1321N1 cells [101]. Compounds **17–20** were claimed to show inhibition at or below 5 μM concentrations, but the IC_{50} values were not reported.

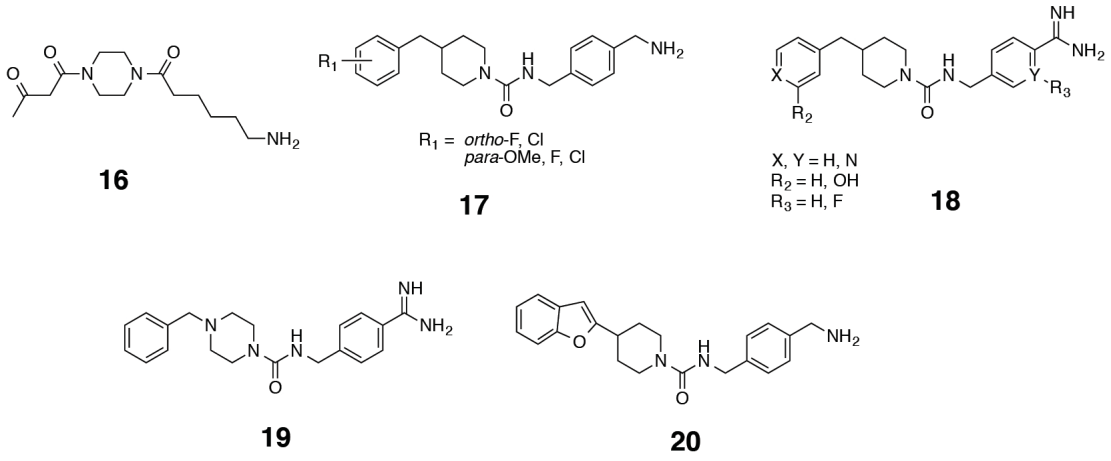

Figure 3. Piperazine and piperidine derivatives reported as PAR2 antagonists.

4.2.2 Quinazoline derivatives

In 2011, Neopharm reported some quinazoline derivatives as micromolar PAR2 antagonists (Figure 4). The compound potencies were assessed under the same conditions reported in the previous patent, where IC_{50} was measured as inhibition of calcium release induced by SLIGKV (2.5 μM) on HCT-15 cells. This patent highlighted four compounds (**21–24**) with **21** containing a *p*-fluorophenyl group and inhibiting PAR2 with IC_{50} 34 μM. Substituting this group with a thiophene (**22**, IC_{50} 17 μM) or cyclopropyl group (**23**, IC_{50} 15 μM) improved antagonist potency by 2-fold. Replacing the bromine on the other phenyl ring with chlorine (**24**) gave a further 2-fold improvement (IC_{50} 8.6 μM). This patent claimed that these antagonists could be useful for the treatment of cardiovascular, gastrointestinal, digestive tract diseases, cirrhosis, atopic dermatitis, asthma, etc [102]. Oral bioavailability and pharmacokinetic profiles of these quinazoline compounds were not reported in the patent, nor was there any data on direct binding or specificity for PAR2.

4.2.3 Benzoimidazole derivatives

NeoPharm also reported a series of benzoimidazole derivatives (**25–27**, Figure 4) in 2012 with inhibition at high micromolar concentrations in the same calcium release assay on HCT-15 cells. These compounds attenuated the calcium response induced by SLIGKV (2.5 μM) with IC_{50} 243, 75 and 77 μM, respectively. They also inhibited β-arrestin recruitment induced by SLIGKV (10 μM) with IC_{50} 95, 339 and 401 μM, respectively. It was noted that these compounds were biased to one particular pathway (calcium or β-arrestin), with **25** being more potent in inhibiting the β-arrestin recruitment, while **26** and **27** were more effective in antagonizing Ca^{2+} release.

Compounds **25** and **26** have been shown to decrease epidermal thickness, PCNA positive keratinocytes, reduce skin barrier disruption and promote skin recovery on an oxazolone-induced chronic dermatitis mice model, indicating possible development into an anti-inflammatory agent to treat skin diseases [103].

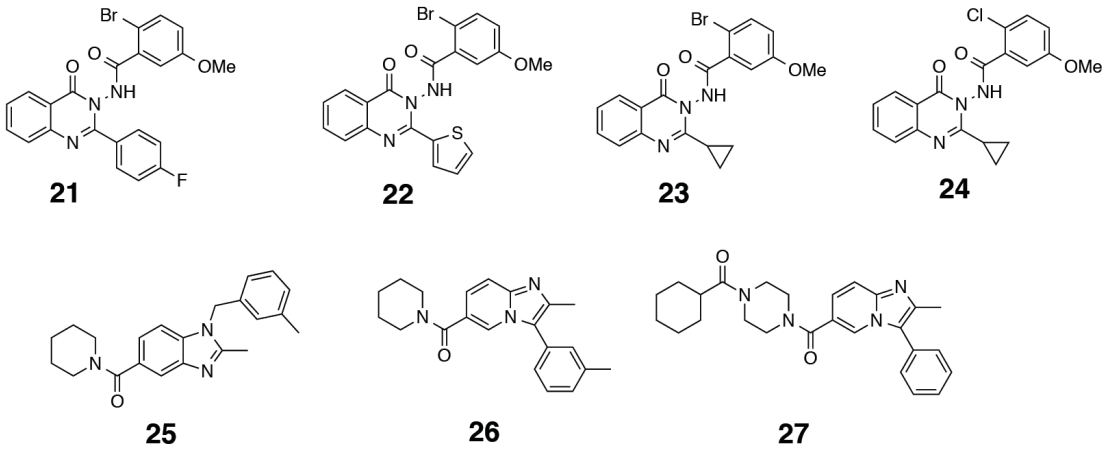

Figure 4. Quinazoline and benzoimidazole derivatives as PAR2 antagonists reported by NeoPharm Co. Ltd. from 2011–2012.

4.2.4 Amidine derivatives

Further SAR studies on the piperidine/piperazine urea derivatives (**17–20**) revealed a group of amidine compounds **28–33** (Figure 5). The piperidine/piperazine motif was replaced with an aromatic ring and the antagonist potency was improved. These compounds were evaluated in the same assay as for **17–20** reported previously, but rat or mouse PAR2 transfected 1321N1 cells were used instead. Amidine compounds **28–32** showed IC_{50} < 1 μM while **33** derivatives were less potent (IC_{50} 1–10 μM) [104]. Although no *in vivo* results were presented in this patent, this series of compounds were claimed to have therapeutic effects for inflammatory diseases such as IBD, ulcerative colitis, Crohn's disease, arthritis, skin inflammation, pain, itch and cancer.

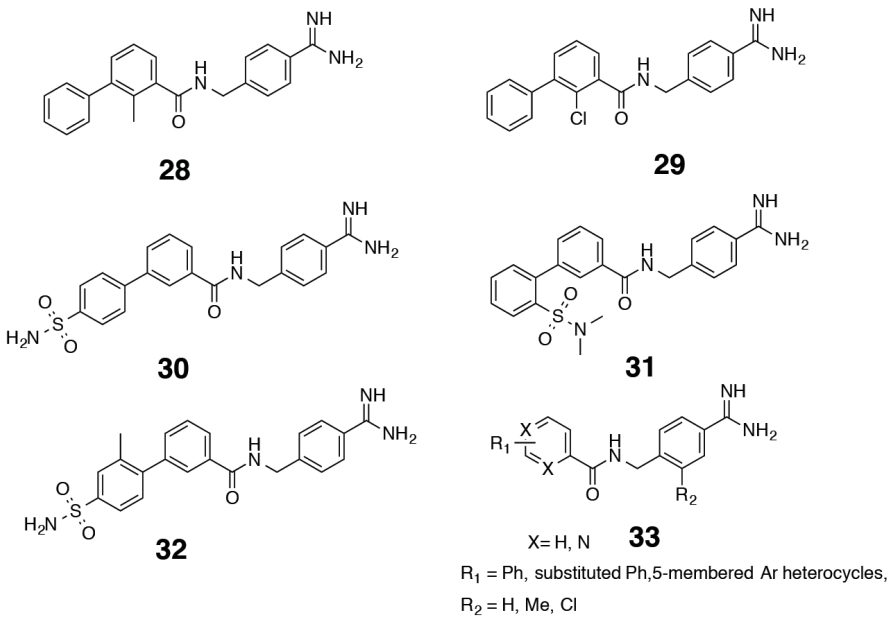

Figure 5. Amidine PAR2 antagonists (**28–33**) reported by Proximagen Ltd. in 2014.

4.2.5 5-Isoxazolyl-Cha derivatives

A library of GB88 (**9**) analogues was reported as PAR2 antagonists in patents, including piperidines and benzylamide compounds containing 5-isoxazolyl-Cha moieties [87, 105]. A series of benzylamide compounds have now published in a journal [98]. For example, compounds **13** and **34** (Figures 2 & 6) inhibited calcium release induced in HT29 by 2f-LIGRLO-NH_2 (1 μM), with IC_{50} 0.5 μM and 0.7 μM, respectively. They also inhibited trypsin (100 nM) mediated calcium release with micromolar activity. These compounds were found to be very stable in rat plasma over 3 h and were more stable than PAR2 peptide agonists (SLIGRL-NH_2 and 2f-LIGRLO-NH_2) in rat liver homogenate. Both **13** and **34** attenuated PAR2-induced paw oedema when given at 5–10 mg/kg s.c. to rats, with **34** being orally active [98, 105]. The reported compounds can modulate the functions of PAR2 in inflammatory, respiratory, cardiovascular and metabolic diseases; including metabolic syndrome,

obesity, type II diabetes, airways/lung inflammation, gastric ulcers, epilepsy, fibrosis, diseases of the skin and subcutaneous tissues, diseases of the muscles, bones and tendons, diseases of the liver, kidney and genitourinary system [87, 105]. These molecules were successfully truncated to give much more potent PAR2 modulators such as agonist **5** (EC_{50} 33 nM, Ca^{2+}, hPAR2 transfected CHO cells)[86, 87], although the scope for PAR2 antagonists derived from or based on such truncated structures has not been disclosed.

4.2.6. Benzothiazine-carboxamide derivatives

In 2015, a group of benzothiazine-carboxamide compounds were reported as PAR2 antagonists (**35–40**, Figure 6) with activity at micromolar concentrations [106]. Antagonist activity of these compounds against SLIGKV-NH_2 (5 μM) was assessed in a calcium release assay on HEK cells (**35**, IC_{50} 1 μM). The phenyl ring on the benzothiazine moiety was found to be important for activity, since replacement by a thiophene, pyridine, chloro-phenyl or methoxy-phenyl ring reduced potency, while displacement of the N-Me with an oxygen atom in **35** abolished antagonist activity. On the other hand, the amide could be replaced by thioamide without loss in potency (**36**, IC_{50} 1 μM). The N-Me could also be replaced with a *para*-halogenated benzyl group to maintain or improve antagonist potency (**38**, IC_{50} 1 μM; **39,** IC_{50} 0.8 μM; **40**, IC_{50} 0.4 μM). These compounds inhibited IL-8 production in human fibroblasts and melanin generation in skin pigment of the C57BL/6 Mel-Ab cell lines, indicating the possibility for PAR2 antagonists in treating skin inflammation. They might also be used as skin-whitening agents [106].

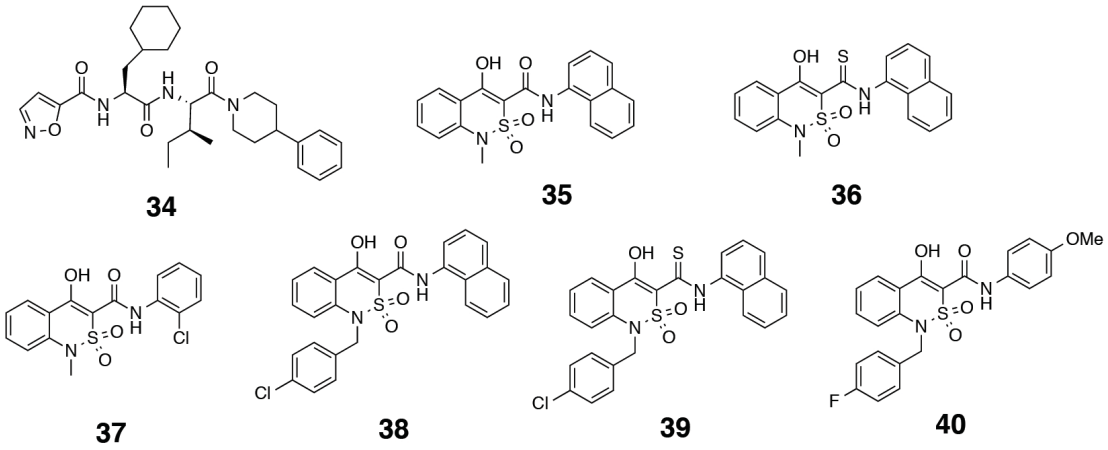

Figure 6. 5-Isoxazolyl-Cha (**34**) and benzothiazine-carboxamide derivatives (**35–40**) as PAR2 antagonists or agonists with sub-micromolar activities.

4.2.7. Imidazopyridazine derivatives

All the PAR2 antagonists (**16–40**) described previously only possess high nanomolar/micromolar/millimolar activity. In 2015, Vertex Pharma reported a series of imidazopyridazine derivatives as antagonists for PAR2, although no data was provided in support of a direct action on PAR2 [107]. Rather they were reported to affect certain activities induced by PAR2 agonists. A decade ago, some pyrazolopyrimidine compounds (e.g. compound **41**) were reported by Sumitomo Pharma to be weakly potent PAR2 antagonists [108]. Modification of this pyrazolopyrimidine compound (**41**) by Vertex Pharma produced imidazopyridazine derivatives (**42–46**) with greater antagonist potency (Figure 7).

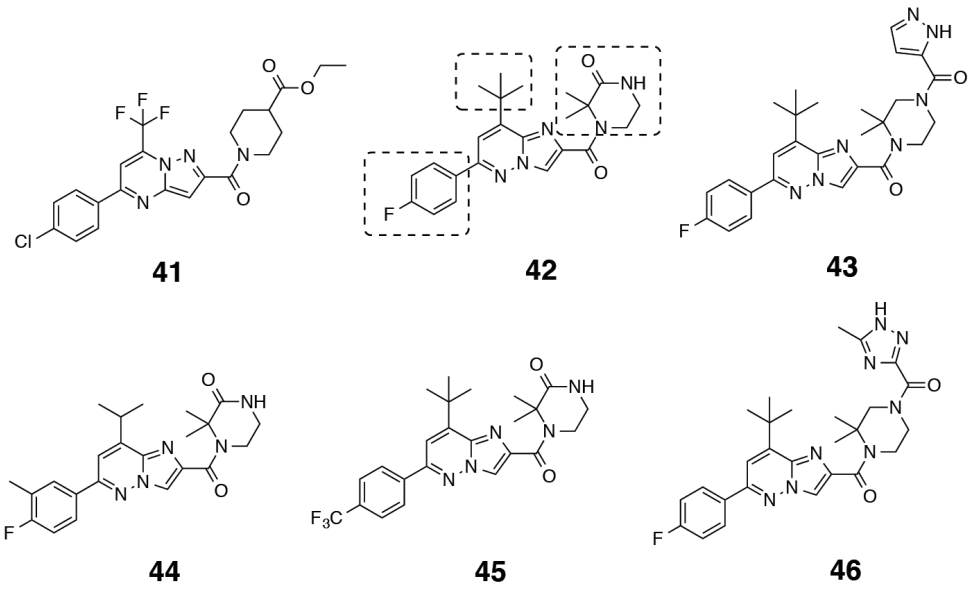

Figure 7. Imidazolepyridazine compounds reported as potent PAR2 antagonists.

Of 600 imidazopyridazine derivatives synthesized, some of the more potent antagonists were evaluated in animal models. It was noted that electron-withdrawing groups were preferred at the *para*-position of phenyl ring, with 4-fluorophenyl giving the optimum potency in most cases. In general, substituting the *tert*-butyl group in **42** with smaller groups such as dimethyl reduced potency, and with a 5-membered aromatic heterocycle, pyridine, or trifluoromethyl group was not well tolerated. However, addition of a dimethyl group on the piperazine ring improved potency in some cases. Also, the piperazine ring could be coupled to a small aromatic heterocycle (imidazole, isoxazole, triazole, pyrazole) without significant loss in potency (**43**, **46**). The biological activity of these compounds was assessed in the commonly used calcium release assay on HT29 cells. The inhibitory potency of these compounds was measured against calcium release induced by SLIGKV-NH_2 (8 μM), trypsin (3.1 U/mL), thrombin (1 U/mL) or UTP (0.6 μM). Compounds that showed promising activities in the calcium assay were tested in rat pharmacokinetic experiments, paw oedema, mechanical hypersensitivity and colitis models. The

potencies of selected compounds are summarized in Table 3. Compounds **42–46** displayed promising pharmacokinetic properties, oral bioavailability and were effective in reducing carrageenan-induced paw oedema, tryptase-induced nociception, and TNBS-induced colitis symptoms in rodent models, suggesting efficacy in inflammatory conditions and pain [107].

4.3 Combination inhibitors

A patent has claimed that inhibition of PAR2 can also be achieved by using a composition with niacinamide (a vitamin B3 compound), *N*-undecylenoyl-L-phenylalanine and a *Laminaria Saccharina* extract [109]. PAR2 expressed on keratinocytes has been shown to regulate pigmentation by transferring melanosomes from melanocytes to keratinocytes via phagocytosis. Thus PAR2 activation will result in hyperpigmentation and eventually lead to skin inflammation. A composition comprises of 5% niacinamide, 1% Sepiwhite (*N*-undecylenoyl-L-phenylalanine) and 1% Phlorogine (*Laminaria Saccharina* extract) inhibited PAR2 activation induced by trypsin in β-arrestin luminescence assay. However, the effective concentration of this composition was not reported. This patent predicted that the *Laminaria Saccharina* extract was capable of boosting the PAR2 inhibitory effect of niacinamide or Sepiwhite and this composition can be applied to human skin surface to reduce hyperpigmentation [109].

4.4 Antibodies

In 2014, the global revenue for monoclonal antibody products was estimated to be $75 billion and FDA approved 5 new antibody-based drugs [110, 111]. Antibodies that

bind to PAR2 might similarly be useful as they can inhibit proteolytic cleavage of the TL and subsequent signaling through G proteins or beta-arrestins. Potent and selective monoclonal anti-PAR2 antibodies might therefore ameliorate PAR2-mediated pathophysiological conditions.

One patent [112] directed at antibodies for PAR2 from Regeneron Pharmaceuticals described the generation of fully human monoclonal anti-PAR2 antibodies, using their proprietary VelocImmune® mice. These mice were genetically humanized by precisely replacing mouse heavy and kappa light chain variable region gene repertoire with human germ-line variable sequences [113, 114]. To generate human monoclonal anti-PAR2 antibodies, VelocImmune® mice were immunized with the human peptide sequence ^{27}GTNRSSKGRSLIGKVDGT^{45}. A series of binding, selectivity and *in vitro* cell assays resulted in monoclonal antibody H4H581P, which inhibited PAR2-induced NF-κB activation at nM concentrations against trypsin, kallikrein, tryptase and factor Xa induced PAR2-mediated NF-κB measured by a luciferase reporter assay. Using matrix assisted laser desorption ionization time of flight (MALDI-TOF) and alanine scan experiments, H4H581P was shown to bind to Val42 and Asp43 of PAR2. In a transgenic mouse model expressing human PAR2, subcutaneous administration of H4H581P (25mg/kg) reduced trypsin- or tryptase- induced pruritus.REF

Amgen described the generational of monoclonal anti-PAR2 antibodies in two related patents [115, 116]. Using the peptide sequence ^{28}TNRSSKGRSLIGVDGTS^{46} as the immunogen, seven monoclonal antibodies were selected and tested for the ability to inhibit trypsin-induced calcium mobilization in HCT-116 and PAR2-transfected

KNRK cells. The most potent antibody, named 47.7, showed IC_{50} 2 nM in HCT-116 and 48 nM in PAR2-transfected KNRK cells respectively. In contrast, a commercially available PAR2 antibody (SAM11) from Santa Cruz Biotechnology showed only slight inhibition (~10%) of trypsin-induced calcium mobilization at 1 μM.

In separate patents [117, 118], Amgen also described anti-PAR2 antibodies that bind to different regions of PAR2. The most potent monoclonal antibody, 1A1 was evaluated in two different *in vivo* carrageenan/kaolin-induced rat knee and paw arthritis models. In a knee arthritis model, carrageenan/kaolin and 1A1 were intra-articularly injected into each knee and 1A1 showed reduction in knee thickness and visual scoring from 6–72h after administration. In the paw arthritis model, 1A1 was administered intra-peritoneally 18h prior to subcutaneous injection of carrageenan into the plantar region of the hind paw. Consistent with the arthritis observations, 1A1 reduced paw oedema and proinflammatory cytokines (IL-6, IFNγ and oncostatin M) in treated paws as compared to untreated arthritic paws.

Instead of utilizing a PAR2 specific immunogen, Boehringer Ingelheim used antibody phage display to screen for Fab fragments that were capable of recognizing the N-terminus of PAR2 [119]. Further affinity maturation and codon optimization of the lead Fab fragment resulted in three high affinity (30–200 pM) PAR2 antibodies that bound to the N-terminus of PAR2. These antibodies inhibited trypsin-induced intracellular calcium (IC_{50} 0.4–1.8 nM) in several human, mouse and rat cells. In the *in vivo* mouse model of delayed type hypersensitivity, the antibodies dose-dependently reduced 50% of footpad swelling at 10mg/kg.

4.5 Pepducins

Kuliopulos *et al* disclosed the rational design of pepducins (lipidated peptides) based on the first and third intracellular loop sequences, and fifth and sixth intracellular loop sequences of PAR2 [60, 120]. Based on the sequence of intracellular loop 3 of PAR2, 8 different *N*-palmitoylated peptides were synthesized. The full-length wild-type pepducin showed very little antagonist activity against 100 μM SLIGRL, however replacement of Arg to Ser, termed P2pal-18S, showed effective antagonism without detectable agonist activity. Chemotaxis experiments also demonstrated that P2pal-18S selectively blocked trypsin-induced neutrophil migration which was selective to PAR2 over PAR1, PAR4 and CXCR2. In the *in vivo* mouse model of inflammatory paw oedema, subcutaneous injection of P2pal-18S (10mg/kg) reduced 50% of paw swelling in carrageenan/kaolin-induced paw oedema and 85% in SLIGRL-induced oedema. Further histological analysis of mouse footpads showed 60% reduction in the infiltrating immune cells as compared to inflamed paws.

5. Conclusions

In the past six years, several new PAR2 agonists and antagonists (peptidic, non-peptidic, antibody) have been claimed in patents and can evidently modulate the functions of PAR2. Some of these modulators were able to activate or inhibit PAR2 actions in animal models of disease, such as airways inflammation, dermatitis, arthritis, colitis, metabolic disorders, pain or cancer. Most PAR2 agonists disclosed were peptides, with relatively few nonpeptidic small molecules reported or claimed to date. On the other hand, most claimed PAR2 antagonists were small molecules with very few peptidic antagonists. Some of these antagonists have been found to inhibit

protease-induced PAR2-mediated functions *in vivo* and might spark further development towards clinical trials. Optimizing potency, PAR2-specificity, signaling bias for different intracellular activation pathways, and pharmacokinetic properties could lead to drug development candidates for validating PAR2 modulation as a viable therapeutic approach in the clinic.

6. Expert opinion

Evidence from several PAR2 knockout studies has indicated that PAR2 is involved in multiple physiological and pathological conditions [2, 5, 21]. Efforts towards developing PAR2-selective modulators have revealed some effective strategies for realizing pharmacologically active compounds. However, effective and selective drugs for modulating PAR2 has been challenging and as yet no small molecule modulators have successfully progressed to clinical trials.

There are some significant challenges for bringing PAR2 modulators to the clinic, since PAR2 has beneficial properties in normal physiology while being detrimental in certain disease settings. PAR2 is now known to be influenced by differential biased signaling induced by different proteases and small molecule ligands, and the functions of PAR2 appear to be context and cell dependent. Antibodies and protein-based PAR2 modulators appear less likely to be useful in dissecting out the therapeutic advantages predicted to accompany selective or biased modulation of signaling pathways. On the otherhand, pathway selective modulation of PAR2 using small molecules appears to be more feasible [10, 15] and might be usefully exploited in deriving pharmaceuticals that can simultaneously attenuate disease while still conferring protective PAR2-mediated benefits to cells and tissues. This brings a degree of complexity to the evaluation of ligand functions, with an apparent need to examine multiple signaling cascades, cell types and *in vivo* assays, for differential activating or inhibiting properties of each ligand and this has slowed the development of PAR2-directed modulators. If pathway selective modulation indeed proves to be important, these

additional efforts could realize superior PAR2 modulators with greater benefits in vivo.

From 2010–2015, a few potent peptidic and non-peptidic PAR2 agonists were reported in journals and a patent, with the large lipid peptide **3** being the most potent compound (EC_{50} ~1 nM, Ca^{2+}, 16HBE14o- cells) [85]. However, **3** and small molecule agonist **6** have some liabilities including poor solubility. AY77 (**5**, EC_{50} 33 nM, iCa^{2+}, CHO-hPAR2) was recently been reported to be the smallest, most ligand-efficient PAR2 agonist that activates the calcium pathway via PAR2 [86, 87]. Not many potent PAR2 antagonists have been described recently. Most PAR2 antagonists patented to date have been small molecules with high nM or high-micromolar potencies (**7–15**, **16–40**), while recently claimed PAR2 antagonists (**42–46**) have yet to be shown to be specific for PAR2 [107]. These imidazopyridazine antagonists inhibited both SLIGKV- and trypsin-induced calcium release on HT29 cells but not PAR1 induced activities. They had oral bioavailability (F% >50) and showed some anti-inflammatory activity in rodents. No evidence however has been reported yet for their direct binding to, or action through PAR2, or for differential effects on different PAR2 signaling pathways.

While some progress has been made in profiling the therapeutic potential of PAR2 antagonists, fewer studies have examined the therapeutic potential of PAR2 agonists and further research is needed to explore this potential. There has been some controversy about the roles of PAR2 being pro- or anti-inflammatory. Some reports have suggested that PAR2 activation is protective in the airways [40, 41, 121] and other tissues, which may be associated with G-protein dependent signaling [46]. Pro-

inflammatory responses caused by PAR2 activation have alternatively been associated with β-arrestin dependent signaling [46]. A biased PAR2 agonist that only signals via certain G-proteins or only via β-arrestins would be a useful probe for understanding context-dependent PAR2 activation in both normal physiology and disease settings.

Not many potent and pathway-selective ligands have been developed for PAR2 to date. Most PAR2 ligands reported in this review have only been evaluated in the PAR2-induced calcium pathway and only in one or a few cell types. There is a need to more widely investigate PAR2 ligand profiles in multiple PAR2-associated signaling pathways, multiple cell lines and multiple disease models to better understand the pharmacology of PAR2 modulation. A worthy goal appears to be not only to develop PAR2-selective and potent modulators with few off-target effects, but also to develop pathway-selective ligands for PAR2 to minimize on-target but off-pathway side effects. Thus drug development for PAR2 may not only require on-target selectivity, but also on-pathway selectivity, in order to realize the full potential of this receptor in modulating particular diseases.

In conclusion, the discovery of PAR2 modulators as new therapeutic agents has presented a number of new challenges for GPCR drug development. There is no endogenous agonist ligand to use as a starting point for drug development. The finding of ligand-induced biased signaling for both protease agonists and small molecule ligands presents further challenges as well as new opportunities. In the past six years, not many potent PAR2 ligands were reported in either patents or the scientific literature. However, given the promise of PAR2 and the new findings of biased signaling ligands, there appear to be exciting opportunities on the horizon in

this field. It seems likely that PAR2 modulators will progress into the clinic over the next few years for several disease conditions.

Declaration of interest

MKY, JL, LL and DPF are named inventors on several patent applications involving PAR2 agonists and antagonists owned by the University of Queensland. No other competing interests.

Article highlight box

- PAR2 is associated with inflammatory, cardiovascular, gastrointestinal, respiratory and metabolic diseases, highlighting it as a potentially valuable therapeutic target. It is also associated with pain, itch, wound healing and cancer metastasis.
- Patents reporting new PAR2 agonists, antagonists, antibodies and pepducins from 2010–2015 are examined.
- Structure-activity relationships and biological activities of PAR2 modulators *in vitro* and *in vivo* as described in patents are reported.
- PAR2 modulators have been found to be beneficial for treating animal models of disease.
- Biased ligands for PAR2 show promise for modulating different signaling pathways that may be linked to different diseased states.

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Table 1. Summary of PAR2-induced or -related disease models and therapeutic effects of PAR2 antagonism/knockout (KO).

Disease model	PAR2 agonist(s)	PAR2 antagonism	Therapeutic effects	References
Mouse model of arthritis induced by destabilisation of medial meniscus	N.A	PAR2 antibody (SAM11), PAR2 KO	Inhibited cartilage erosion, swelling and arthritis pathology	[55, 56]
Synovial fibroblasts isolated from patient with rheumatoid arthritis	SLIGRL-NH_2	ENMD-1068, PAR2 siRNA	Inhibited invasion and proliferation of synovial fibroblasts and TNF secretion	[57]
Mouse model of oxazolone-induced dermatitis	N.A	PAR2 KO	Reduced oedema and inflammatory cells infiltration	[58]
Rat model of paw oedema	2f-LIGRLO-NH_2	GB88	Prevented collagen loss, mast cell degranulation and macrophages accumulation	[59]
Mouse model of paw oedema	Mast cell tryptase SLIGRL-NH_2	P2pal-18S	Inhibited edema and myeloperoxidase activity	[60]
Mouse model of DSS-induced colonic inflammation	N.A	PAR2 KO	Reduced edema, fecal blood and bowel thickness	[61]
Human colonic epithelial cells (HT29)	2f-LIGRLO-NH_2 SLIGKV-NH_2	PAR2 siRNA	Reduced cytokines-induced apoptosis	[62]
Rat model of colonic inflammation	SLIGRL-NH_2	GB88	Inhibited mucin depletion and colon wall thickness	[63]
Mouse model of ovalbumin-induced airway inflammation	N.A	PAR2 KO	Inhibited eosinophil infiltration and hyperreactivity	[64]
Mouse model of asthma	German cockroach feces extracts	PAR2 KO	Reduced airway inflammation and immune cells recruitment	[65]
Mouse model of asthma	House dust mite extracts	PAR2 KO	Reduced airway inflammation and eosinophil accumulation	[66]
Mouse model of cancer nociception	Trypsin	PAR2 KO	Inhibited cancer-induced allodynia	[67]

Mouse model of hyperalgesia	SLIGRL-NH_2 Trypsin Tryptase	PAR2 KO	Reduced thermal and mechanical hyperalgesia	[68]
Mouse model of hyperalgesia	Neutrophil elastase	PAR2 KO	Reduced edema and mechanical hyperalgesia	[69]
Mouse model of bone cancer-induced hyperalgesia	N.A	FSLLRY-NH_2 PAR2 KO	Reduced mechanical allodynia and thermal hyperalgesia	[70]
Mouse model of pancreatitis-induced hyperalgesia	2f-LIGRLO-NH_2	PAR2 KO	Agonist reduced hyperalgesia and allodynia in WT mice	[71]
Mouse model of diet-induced metabolic dysfunction	N.A	PAR2 KO	Prevented weight gain, insulin resistance and inflammation	[44]
Rat model of diet-induced metabolic dysfunction	NA	GB88	Prevented weight gain, insulin resistance and inflammation	[45]
Mouse model of retinal angiogenesis	SLIGRL-NH_2	PAR2 KO	Agonist improved retinal vascularisation and angiogenesis in WT mice	[72]
Mouse model of mammary tumour	N.A	PAR2 KO	Delayed development and metastasis of tumor	[73]
Mouse model of mammary tumour	N.A	PAR2 KO	Delayed development and metastasis of tumor	[74]

Table 2. Summary of the inhibitory activities of small molecule PAR2 antagonists (reported in patents 2010–2015) against different PAR2 agonists, assessed in an intracellular calcium release assay.

Compd	Patent number	Organization	Derivatives	Cell line	IC_{50} (μM)	Agonist used	Possible therapeutic applications
16	KR2010038919	NeoPharm Co. Ltd.	Piperazine/piperidine compounds	HCT-15	~1200	SLIGKV (2.5 μM)	Atopic dermatitis
21–24	KR2011130259	NeoPharm Co. Ltd.	Quinazoline compounds		9–34		Cardiovascular, gastrointestinal, digestive tract diseases, cirrhosis, atopic dermatitis, asthma, etc
25–27	WO2012026765 WO2012026766	NeoPharm Co. Ltd.	Benzoimidazole compounds		75–243		Skin diseases
17–20	WO2012101453	Proximagen Ltd.	Piperidine/Piperazine urea compounds	Human PAR2 transfected 1231N1	< 5	Trypsin (EC_{50} conc.)	Multiple inflammatory diseases and cancer
28–33	WO2014020350 WO2014020351	Proximagen Ltd.	Amidine compounds	Rat or mouse PAR2 transfected 1231N1	< 1		
13, **34**	WO2013013273 US20150038402	The University of Queensland	5-Isoxazolyl-Cha compounds	HT29	0.4–4.0	2f-LIGRLO-NH_2 (1.0 μM), trypsin (0.1 μM)	Inflammation, metabolic syndrome, obesity, type II diabetes, fibrosis, cardiovascular diseases, etc.
35–40	KR2015044675	AmorePacific Corp.	Benzothiazine-carboxamide compounds	HEK	0.4–1.0	SLIGKV (5 μM)	Skin inflammation
41–46	WO2015048245	Vertex Pharm.	Imidazopyridazine compounds	HT29	0.001–0.01	SLIGKV (8 μM), trypsin (3.1 U/mL)	Inflammation and pain

Table 3. Pharmacological properties of selected imidazopyridazine derivatives [107].

	42	**43**	**44**	**45**	**46**
IC_{50} (nM, Ca^{2+}, HT29) vs SLIGKV	1.4	0.7	7.5	2.2	0.5
IC_{50} (nM, Ca^{2+}, HT29) vs Trypsin	2.3	0.9	10.2	2.0	0.8
IC_{50} (nM, Ca^{2+}, HT29) vs Thrombin & UTP	>400	>400	>3500	>1000	>1000
$T_{1/2}$ (h)*	57	4	5	78	7.0
Cl_p (mL/min/kg)*	0.7	10.9	4.8	1.1	3.4
Vss*	3.2	3.3	1.6	6.8	1.6
F %*	66	55	70	76	62
Inhibition of carrageenan-induced paw oedema*	50% (6mg/kg)	55% (5mg/kg)	51% (6mg/kg)	19% (20mg/kg)	50% (10mg/kg)
Inhibition of tryptase-induced nociception*	40% (10mg/kg)	52% (5mg/kg)	43% (6mg/kg)	N.A.	23% (10mg/kg)
Inhibition of TNBS-induced colitis**	21% (15mg/kg)	N.A.	N.A.	N.A.	24% (15mg/kg)

Vss *Volume of distribution at steady-state;* N.A. *not available*
* Experiments performed in rats; ** in mice