Recent Advances in Synthesis of PAR Ligands as Therapeutic Strategy for Inflammatory Diseases

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Abstract: Several studies have been published on discovering involvement of PARs receptors in a number of disease states, including cancer and inflammation of the cardiovascular, respiratory, musculoskeletal, gastrointestinal and nervous systems. This mini-review will focus on recent advances in the synthesis of PAR ligands highlighting their therapeutic potential in the treatment of various inflammatory diseases.

Key Words: Protease activated receptors, inflammation, agonist, antagonist, peptide-mimetic, small-molecule.

INTRODUCTION

Serine proteases, such as thrombin and trypsin, can regulate target cells by cleaving and activating G-protein coupled protease activated receptors (PARs) [1-10]. These receptors, named PARs 1 to 4 in order of their discovery, show an original mechanism of receptor triggering that distinguishes them from other seven transmembrane GPCRs. This entails the proteolytic cleavage of a specific site within the receptor N-terminus to reveal a cryptic tethered ligand that binds to and activates the receptor [11-13]. A number of endogenous proteases are able to activate members of the PAR family: thrombin cleaves the N-terminal domain of PAR1, PAR3, and PAR4; trypsin activates PAR2, PAR4, and to a lesser extent PAR1; cathepsin G activates PAR4; and mast cell tryptase can activate PAR2 [14]. Like trypsin and thrombin, exogenous proteinases, such as pathogen proteinases, can either activate or disarm PARs, by removing the N-terminal activation site [15, 16]. In addition, three of the PARs (PAR1, PAR2, and PAR4, but not PAR3) are also activated by short synthetic peptides, Fig. (**1**), derived from the sequences of the proteolytically revealed tethered ligand [1, 17]. These peptides have been very useful tools to better understand the physiological role of PARs. PAR-selective activating peptides, in fact, have allowed exploration of the effects of PAR activation on targeted cells and tissues, without the need of the less selective protease activation. Numerous studies, performed in order to explore the pathophysiological role of PARs, have demonstrated their critical involvement in coagulation, inflammation, and vascular homeostasis. Most of the obtained results are related to PARs as potential targets for treating cardiovascular diseases such as thrombosis, atherosclerosis, and restenosis [18-23]; PARs, indeed, are dramatically important in maintaining vascular integrity. Here we summarize the most recent evidence of the important participation of PARs in the crosstalk between coagulation and inflammation processes. The main part of the mini-review is pointed on reporting the development in the preparation and biological evaluation of PAR ligands; the classification of the molecules is based firstly on the specific target and secondly on the chemical structure. In particular we analyze: i) heterocycle-based peptide-mimetic PAR1 antagonists; ii) small-molecule compounds as PAR1 antagonists; iii) peptide-mimetic PAR2 agonists; iv) small-molecule compounds as PAR2 agonists and antagonists; v) peptide-mimetic PAR4 agonists and antagonists; vi) small-molecule compounds as PAR4 antagonists; vii) pepducins as PAR4 antagonists.

PARS AND INFLAMMATION

It is not yet well defined whether the role for PAR activation relates to pro-inflammatory effects, a protective role or both. However, numerous studies indicate PARs as prime candidates for the crosstalk between inflammation and coagulation. PAR1, the first member of this family to be cloned, mediates several effects of thrombin other than platelet activation, especially inflammation. *In vivo*, PAR1 agonists can induce inflammation when injected into the paw, the liver, or the brain of rodents [24-26]. PAR1 activation induces changes in vascular tone, increased vascular permeability, and granulocyte chemotaxis [15, 27]. In lung epithelial cells, PAR1 (but also PAR2 and PAR4) stimulate the release of pro-inflammatory cytokines (IL-6 and IL-8), proposing protease activated receptors as important target in developing novel therapeutic strategies in various lung diseases. A strong impact of PAR1, the main protease receptor expressed by lung fibroblasts, in the development of lung fibrosis was shown in PAR1 deficient mice, when fibrosis was induced by intratracheal bleomicin instillation. Thus PAR1 has emerged as exciting target for clinical intervention in fibrotic lung diseases; while PAR2 seems to contribute to allergic airway inflammation [28, 29]. Moreover, PAR1

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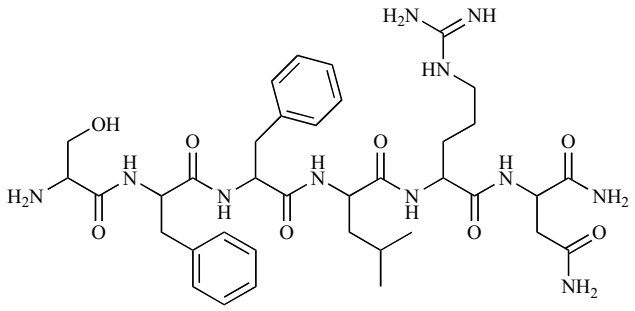
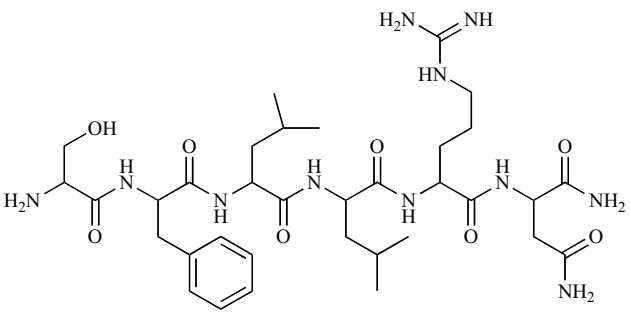
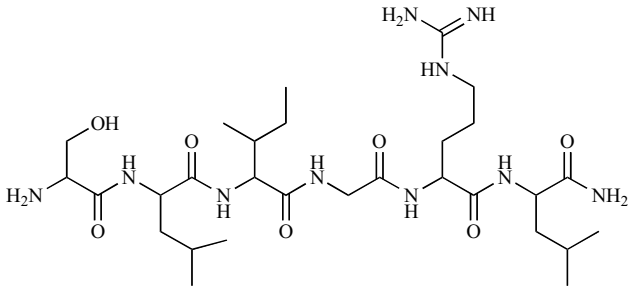


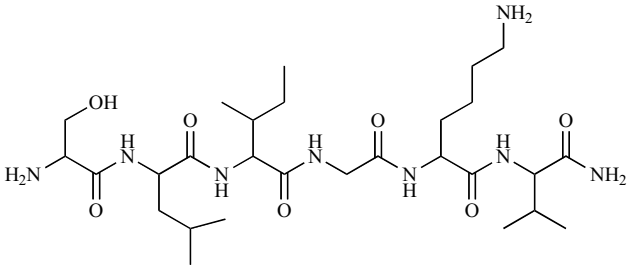
PAR1-AP murine (SFFLRN-NH_2)



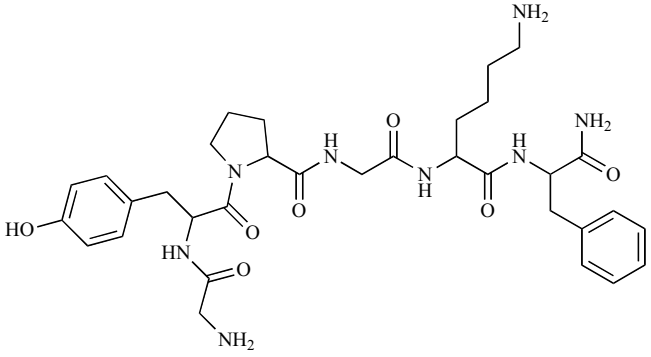
PAR1-AP human (SFLLRN-NH_2)



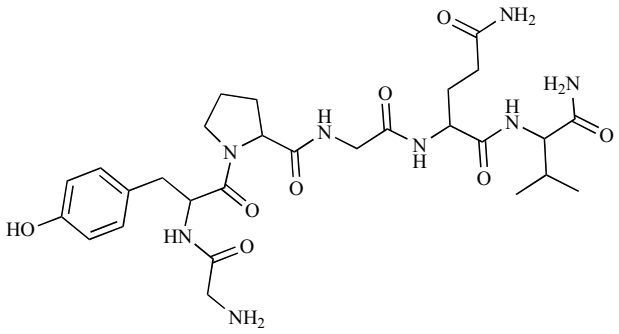
PAR2-AP murine (SLIGRL-NH_2)



PAR2-AP human (SLIGKV-NH_2)



PAR4-AP murine (GYPGKV-NH_2)



PAR4-AP human (GYPGQV-NH_2)

Fig. (1). Structures of murine and human PAR activating peptides.

plays a crucial role in the pathogenesis of experimental colitis, supporting the notion that PAR1 inhibition may be beneficial in the context of inflammatory bowel disease (IBD) and possibly in other chronic intestinal inflammatory disorders [14].

PAR2 involvement in syndromes with a strong inflammatory component has been well documented. These include colitis, gastritis, pancreatitis, asthma and pulmonary disease, and arthritis [30-35]. PAR2 mediates physiological actions through a neurogenic mechanism. Consequently, it would be expected that ligands able to activate PAR2 should exacerbate most nociceptive and inflammatory processes. Indeed, there is still some controversy on this point because it has been demonstrated that PAR2 also exerts a protective effect in many settings such as, in particular, asthma and lung inflammation [34, 36, 37]. This double role could lead to the possible therapeutic use of PAR2 agonists while the application for PAR2 antagonists, that have already demonstrated their potential in relieving symptoms in models of rheumatoid arthritis, appears clearer [32, 35].

PAR4 was identified as the fourth protease-activated receptor that can be activated not only by thrombin but also by trypsin and cathepsin G. The highest levels of PAR4 mRNA were detected in lung, pancreas, thyroid and intestine. Recent reports have focused attention on the contribution of PAR4 to long-term cellular responses, demonstrating that PAR4 stimulation, on primary cultured mouse lung alveolar epithelial cells, produced a phospholipase Cβ-dependent mobilization of Ca^{2+} from intracellular stores (generating an initial peak) followed by Ca^{2+}-entry through store depletion-operated Ca^{2+} pathway (generating a slowly decaying delayed phase) [38]. In addition, the elevation of PAR4 protein level was detected in rabbit lung treated with endotoxin [39]. The important role played by this receptor subtype in inflammatory disorders has been further confirmed by the beneficial effects obtained by blocking PAR4 with cell-penetrating peptides (pepducins), that disrupt G-protein coupling [40].

HETEROCYCLE-BASED PEPTIDE-MIMETIC PAR1 ANTAGONISTS

Thrombin cleavage at the PAR1 N-terminus exposes a receptor-linked activating ligand, such as the hexapeptide SFLLRN. Notably, synthetic SFLLRN-NH_2, usually named PAR1-Activating Peptide, PAR1-AP, Fig. (**1**), exerts full agonist activity, although its potency on platelets is 1000-fold less than thrombin's. Design and preparation of peptide-mimetics or small organic molecules with PAR1 antagonist properties has been very challenging since the tethered ligand binding mechanism is energetically preferred. It is very difficult to compete against this intramolecular binding with a small-molecule ligand, moreover very few information about conformation of the receptor is available, this means that a highly empirical approach has been used so far. Although much experimental evidence supports the crucial involvement of PAR1 in different inflammatory diseases, to date, most of the published studies on PAR1 antagonists concern their employment as antithrombotic agents. In this context other challenges arose from the absence of PAR1 in platelets of different test animals (e.g., rats and dogs) and the presence of additional thrombin receptors, PAR3 and PAR4, in different species [41].

Early PAR1 antagonists were designed on the basis of the SFLLRN motif of the tethered ligand of the PAR1. Functional assays such as platelet aggregation, GTPase turnover, proliferation assays using thymidine incorporation, and intracellular calcium mobilization were used to identify agonists. Extensive structure-activity relationships (SAR) studies, started from the identification of a "three-point model", constituted by the ammonium group, the center of the benzene ring (Phe residue) and the central carbon of the guanidine group (Arg residue) in conjunction with different molecular templates such as benzene, naphthalene, benzimidazole and indole, allowed the discovery of potent heterocycle-based peptide-mimetic PAR1 antagonists with general structure reported in Fig (**2**), characterized with six variables (R_1-R_6).

From this screening the indole-based **1** (RWJ-56110) and indazole-based **2** (RWJ-58259) were found, both equipotently inhibiting thrombin induced platelet aggregation with IC_{50} values of 340 and 370 nM, respectively [42-44]. Studies with **3** (RWJ-54003), another related indole-based compound (IC_{50} value for thrombin-induced platelet aggregation of 0.9 μM [40]), in a rat model of cirrhosis showed protective effects of the PAR1 antagonist against liver fibrosis development [45]. Recently a new series of peptide-mimetic antagonists containing novel heterocyclic scaffolds, such as 2-methyl-indole and 1,4-benzodiazepine, was described. The pharmacological activity was assessed using human platelet aggregation induced by PAR-1AP in order to test the antagonist potency. Compound **4**, Fig. (**2**), differs from **3** (RWJ-54003) only because of the presence of a methyl group in position 2 of the 6-aminoindole moiety; this small structural modification shifted the biological activity towards a more potent derivative (IC_{75} analysis gave 0.1 μM for the reference compound **3**, and 0.02 μM for compounds **4**) [46].

SMALL-MOLECULE COMPOUNDS AS PAR1 ANTAGONISTS

In 2005, high affinity, orally active, low molecular weight non-peptide PAR1 antagonists, based on the natural product himbacine (**5**, Fig. (**3**)), were reported [47]. The original interest in himbacine was for its antimuscarinic properties, for central nervous system indications. A total synthesis of himbacine was described in 1996 and several analogues were synthesized [48]. Initial SAR studies were directed at optimizing the substitution pattern on the pyridine ring. The newly synthesized compounds were tested by *in vitro* binding assays, carried out using purified human platelet membranes as PAR-1 source and tritiated high-affinity thrombin receptor activating peptide, alanine-*p*-fluorophenylalanine-arginine-cyclo-hexylalanine-homoarginine-[^{3}H]phenylalanine amide ([^{3}H]haTRAP, K_d = 15 nM), as ligand [48]. The obtained results indicate that the presence of an alkyl group at the C-6 position of the pyridine ring, compounds

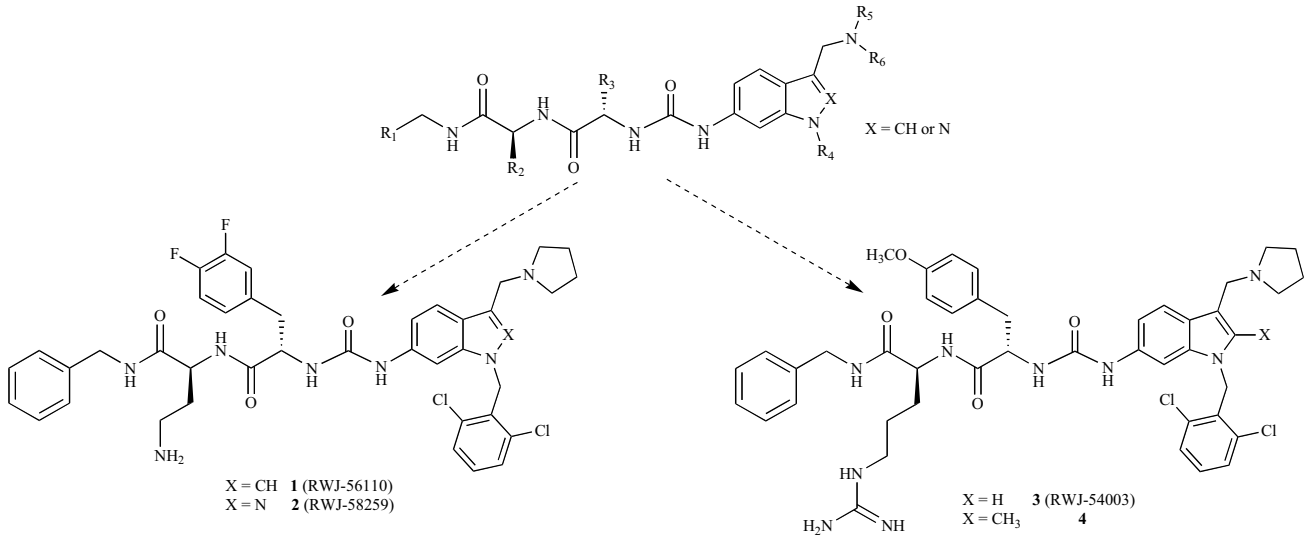


Fig. (2). Indole- and indazole-based peptide-mimetic PAR1 antagonists.

6-9, Fig. (**3**), is preferred over alkyl groups at other positions with ethyl group furnishing the best PAR1 affinity.

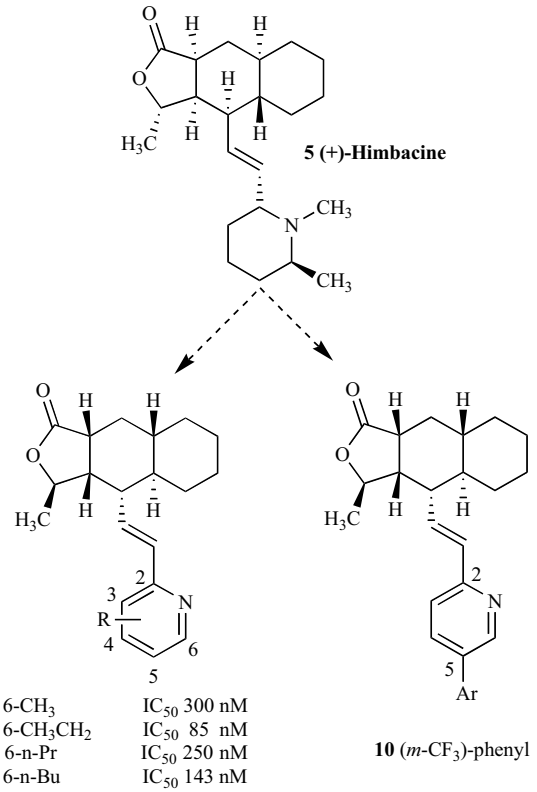


Fig. (3). Himbacine derived PAR1 antagonists.

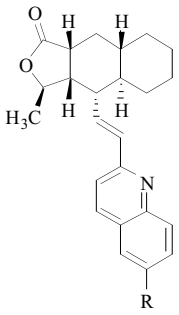
In an effort to further characterize the PAR1 antagonist profile of the himbacine series, **6** and **7** were resolved using chiral HPLC. The (+)-enantiomer was found to be approximately 10 times more active than the (-)-enantiomer in each case. In the radioligand binding assay, (+)-**7a** showed a K_i of 12 nM against PAR1. In the haTRAP induced human platelet aggregation assay, this compound showed dose-dependent inhibition of aggregation with an IC_{50} of 70 nM. This compound was further evaluated in an *ex vivo* platelet aggregation assay in conscious cynomolgus monkeys after iv infusion (10 mg/kg, 30 min). Nearly complete inhibition of platelet aggregation induced by exogenously added haTRAP to the plasma drawn from the drug treated group was noted for 2 h. However, this compound showed poor oral bioavailability in rats. To achieve compounds with better pharmacokinetic properties, C-5 aryl substituted derivatives were examined.

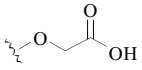
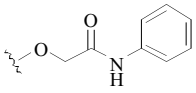
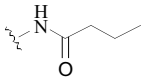
In the radioligand binding assay, **10** showed a K_i of 2.7 nM against PAR1; moreover it showed robust inhibition of thrombin-induced platelet aggregation with an IC_{50} of 44 nM and haTRAP induced platelet aggregation with an IC_{50} of 24 nM, whereas it showed no inhibition of ADP and collagen induced aggregation of platelets. Detailed pharmacokinetic studies were performed showing an excellent bioavailability in rats (30% at a dose of 10 mg/kg; half-life was 3.2 h following iv administration) and in cynomolgus monkeys (50%; half-life was 12.4 h after iv administration). Compound **10** did not affect clotting parameters (PT, prothrombin time; APTT, activated partial thromboplastin time), confirming that its mechanism of action is not by active site inhibition of thrombin or other coagulation proteases; moreover it was selective against a number of GPCRs, ion channels, and was inactive in the PAR2 and PAR4 functional assays. In order to explore the possibility of a synergistic effect between substitutions at positions -5 and -6 of the pyridine ring, and to further refine the previously mentioned lead compounds **7** and **10**, Fig. (**3**), a series of quinoline derivatives were synthesized, Table (**1**) [49]. The initial results showed that substitution at the 6-position of the quinoline ring with an electron-donating group gave the most potent compounds. The 6-methoxy-substituted compound **11** gave comparable binding data (IC_{50} = 15 nM) to lead structures **7** and **10**. A further exploration of the SAR of the 6-position of the quinoline moiety furnished the most potent compound of the series, **12**, with a PAR1 IC_{50} of 6.3 nM. Other compounds of note were **13-15**, and amide **16** all with IC_{50} in the 20–30 nM range. When tested in an *ex vivo* model of platelet aggregation, **13** showed almost complete inhibition 2 h after iv dosing in cynomolgus monkeys. It was not active when dosed orally due to poor oral bioavailability. Surprisingly, **11** and **12** did not show substantial activity in the *ex vivo* model of platelet aggregation, which is presumably due to rapid conjugation and clearance.

A further optimization of this himbacine-derived PAR1 antagonists has led to the discovery of **17** (SCH-530348), Fig (**4A**), that showed K_i = 8.1 ± 1.1 nM in the *in vitro* binding assays, carried out on human platelet membrane-derived PAR-1 receptors using a radiolabeled high affinity thrombin receptor activating peptide ([^{3}H]haTRAP) as ligand [50]. Exploration of the C-7 region of the tricyclic motif furnished **17**, which is characterized by unique properties such as an excellent oral bioavailability in multiple species (33% in rats and 86% in monkeys), high potency in a series of *in vitro* functional assays (inhibition of thrombin-induced platelet aggregation, IC_{50} = 47 nM; haTRAP-induced platelet aggregation, IC_{50} = 25 nM), and potent oral activity in an *ex vivo* cynomolgus monkey model of platelet aggregation (complete inhibition of platelet aggregation is achieved for 24 h post dosing with partial recovery occurring at 48 h at 0.1 mg/kg).

After successfully completing phase-I and phase-II clinical studies, **17** has entered phase-III clinical studies for acute coronary syndrome and secondary prevention of cardiovascular events in high-risk patients [50]. Pyrroloquinazoline-based PAR1 antagonist **18** (SCH-79797), Fig. (**4B**), first reported by Ahn *et al*. in 1999 [51], has shown interesting pharmacological activities. It was able to inhibit haTRAP induced platelet aggregation with an IC_{50} of 56 nM and it was also shown to transiently inhibit platelet aggregation induced by thrombin. A cardioprotective effect, related to its ability in inhibiting PAR1, has been demonstrated; **18**, in fact, is able to attenuate myocardial injury and dysfunction related to myocardial I/R injury when given before or during ischemia [52]. Recently, **18** and **1** have been tested in an *in vivo* angiogenesis model providing direct evidence that PAR1 is involved in the initiation of the angiogenic cascade

Table (1). SAR of Quinoline Derivatives of Lead Compounds 7 and 10



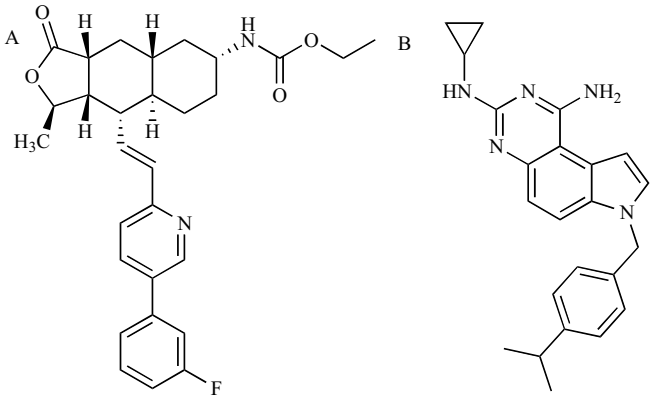
Compound	R	PAR1 IC_{50} (nM)[a]
11	$-OCH_3$	15
12	-OH	6.3
13		29
14		25
15	$-NH_2$	30
16		28

[a]PAR-1 binding assay ligand: [^{3}H]haTRAP, 10 nM (K_d = 15 nM) [48].

[53]. Moreover, **18** has shown a remarkable antiproliferative effect in several cell lines, even though the observation that it was able to slow the proliferation rate of mouse PAR1 null cells as well, suggested that this pharmacological effect was likely not mediated by PAR1 inhibition [54].

PEPTIDE-MIMETIC PAR2 AGONISTS

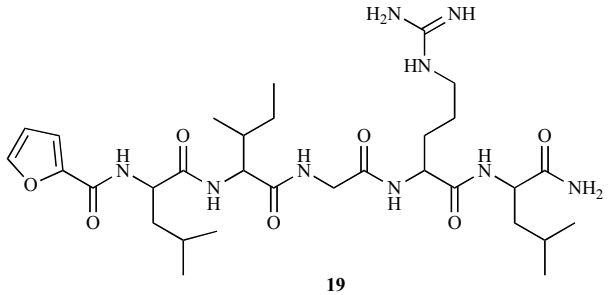
Synthesis of PAR2 ligands has been addressed to both agonists and antagonists. Blocking PAR2 receptors by means of antagonists is of particular interest in order to relieve, for example, inflammatory symptoms in rheumatoid arthritis [32, 35]. Interestingly, PAR2 agonists may also have therapeutic value, in fact it has been suggested that activation of PAR2 could play a relevant role as gastric cytoprotective and/or airway smooth muscle relaxation mediator [34, 36, 37]. As previously stated about PAR1 agonists/antagonists development, the research for PAR2 ligands have started from chemical modifications of short peptides corresponding to the newly exposed N-terminus of the receptor created following cleavage by serine proteases, such as PAR2-AP that



17 (SCH-530348) **18** (SCH-79797)

Fig. (4). Potent heterocyclic PAR1 antagonists.

include the human, SLIGKV-NH_2, and murine, SLIGRL-NH_2, sequences, Fig. (**1**) [55, 56]**.** One of the most potent PAR2 agonist is 2-furoyl-LIGRL-NH_2, **19**, Fig. (**5**), characterized with the replacement of serine at the N-terminus with a furoyl moiety; this compound have shown a potency 10-fold higher than SLIGRL-NH_2 for PAR2 and a remarkable selectivity over PAR1 [57].



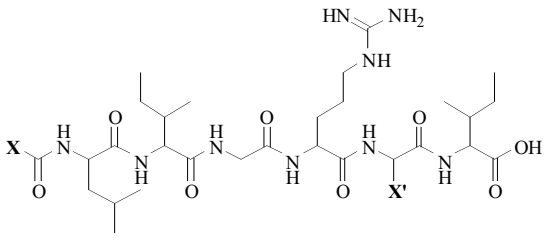
19

Fig. (5). Structure of peptide-mimetic PAR2 agonist, furoyl-LIGRL-NH_2.

In a recent paper [58], pointing to draw a new ligand pharmacophore model, a series of potent PAR2 agonists derived through different modifications within SLIGRL-NH_2 have been described. In this study, it has been found that addition of a seventh residue to the C-terminus, notably, aromatic side-chains like tyrosine (EC_{50} 1.2 μM), homophenylalanine (EC_{50} 1.0 μM), 1-naphthylalanine, (EC_{50} 1.1 μM), 4-fluorophenylalanine, (EC_{50} 2.0 μM), and homotyrosine (EC_{50} 0.6 μM), increased agonist potency by 2- to 6-fold as measured by intracellular calcium efflux.

A previous study from the same authors indicated the potential of carboxyl terminal extensions to SLIGRL-NH_2 to increase the potency of PAR2 agonists. Specifically, extensions using amino acids that are either hydrophobic or introduce additional steric bulk at the *C*-terminal leucine and beyond appear to be effective in enhancing agonist potency, without compromising selectivity for PAR2 over PAR1 [59]. Subsequently, a series of heptapeptides with general formula SLIGRXI-NH_2 were investigated, verifying that introduction of a bulkier residue had a negative effect on agonist activity except when tyrosine (**21**), 4-nitrophenylalanine (**22**), 3,4-dichlorophenylalanine (**23**) and 4-benzo-thienylalanine (**24**) were introduced (see Table **2**) [58]. In these cases, in fact, the derivatives were equipotent with 2-furoyl-LIGRL-NH_2. These results suggest that C-terminal residues at positions 6

Table (2). Intracellular Ca^{+2} Release in HT-29 Cells by Terminal Amidated Heptapeptides XLIGRX'I-NH_2, with Varying Residues X and X'



Compound	X	X'	EC_{50} (μM)[a]
20	Ser	Leu	0.7 ± 0.06
21	Ser	Tyr	0.4 ± 0.1
22	Ser	4-Nitro-Phe	0.4 ± 0.1
23	Ser	3,4-Dichloro-Phe	0.3 ± 0.03
24	Ser	3-benzothienyl-Ala	0.3 ± 0.05
25	2-Benzofuranyl	Leu	0.25
26	2-Naphtyl	Leu	0.36
27	2-Benzothienyl	Leu	0.37
28	2-Indolyl	Leu	0.59
29	2-Pyridyl	Leu	0.26
30	3-Pyridyl	Leu	0.22
31	2-Pyrazyl	Leu	0.29 ± 0.01
32	5-Isoxazolyl	Leu	0.22 ± 0.02
33	4-(2-Methyloxazolyl)	Leu	0.18 ± 0.004
34	2-Furoyl	4-Nitro-Phe	0.2 ± 0.03

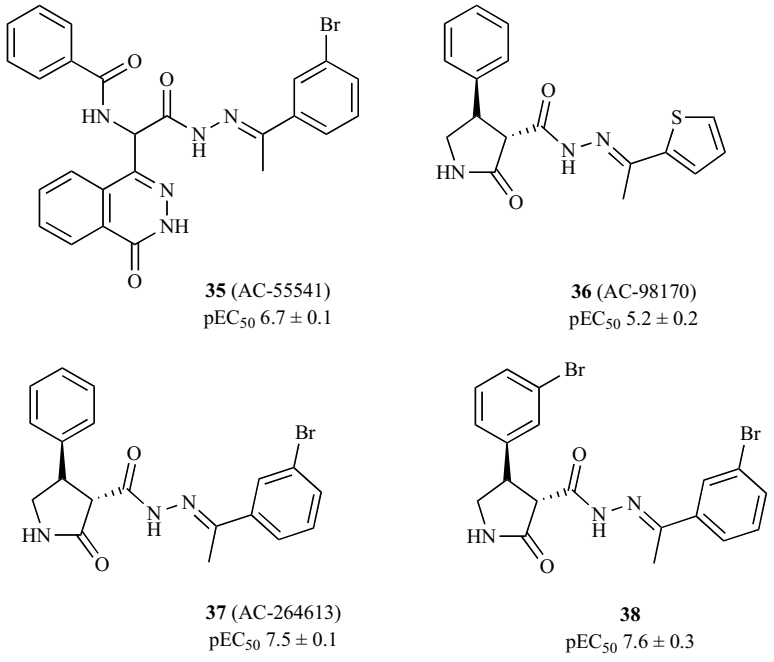
[a]Data from Ca^{2+} release assay [58].

and 7 of SLIGRLI-NH_2 (**20**) may bind in a large hydrophobic pocket of PAR2, and that the interactions between receptor and agonist are predominantly steric. Replacement of N-terminal serine with several aromatic and aliphatic moieties revealed that a number of substitutions other than furan are capable of eliciting similar results. Bulky bicyclic moieties such as benzofuran (**25**), naphthalene (**26**), benzothiophene (**27**), and indole (**28**), in fact, are all tolerated and displaying activity comparable to substitution with furan. Other small aromatic moieties like isoxazole, oxazole, and pyridine (compounds **29**-**33**) are found to be excellent alternatives to serine. No additive effect were observed when the concurrent replacement of the first (Ser) and sixth (Leu) residues of **20** with furoyl and 4-nitro-phenylalanine, respectively, were effected (**34**, EC_{50} 0.2 ± 0.03 μM). Other peptide-mimetic agonists such as *trans*-cinnamoyl-LIGRLO-NH_2 [60] and 2-furoyl-LIGRLO-NH_2 [61] have been developed. In general, the low potency and susceptibility to proteolytic degradation of these compounds are major limitations, particularly for *in vivo* use.

SMALL-MOLECULE COMPOUNDS AS PAR2 AGONISTS AND ANTAGONISTS

Little progress has been made in identifying non-peptidic, non-proteolytic agonists or antagonists of PAR2. From the screening of a chemical library containing more than 250000 small-molecule druglike compounds a number of active structures were identified, Fig. (**6**) [62]. Agonist activity at the human PAR2 has been evaluated utilizing a high-throughput functional screening, such as R-SAT. A focused library around **35,** made by reacting a number of aromatic aldehydes or ketones with the parent hydrazide, revealed that the methyl substituent on the hydrazone was essential for activity whereas hydrogen or higher alkyl groups had a detrimental effect. Although a wide range of substituents in the 3-position of the aryl hydrazone of **35** were beneficial for the activity at PAR2, any substituent in either the 2- or 4-position led to reduced activity. However, the low solubility of **35**, combined with the lack of reasonable building blocks that made difficult the synthesis of analogues shifted the authors to a hit-to-lead optimization of **36**. Starting from a molecular overlay of **35** and **36,** suggesting that substituents *ortho*- or *meta*- in the pyrrolidinone aromatic part should be tolerated as should substituents on the pyrrolidinone nitrogen, preparation of compound **37** (AC-264613; Fig. (**6**)), was accomplished by means of a two step synthetic route starting from commercially available materials. Substituents in the 2- and 3-position of the 4-phenyl of the pyrrolidinone were allowed; in fact, 3-bromo substituted compounds, such as **38**, were highly active even if none of them displayed increased activity toward PAR2 compared to the lead.

Compound **37** was more active than **35** and had a low intrinsic clearance *in vitro* using liver microsomes, but unfortunately, it showed an aqueous solubility as low as compound **35**. However, using surfactants increased the solubility of compound **37** to a satisfying level, making possible a complete pharmacological characterization. Moreover, the enantiomers of **37** were separated using chiral HPLC verifying that the (+)-isomer was responsible for essentially all agonist activity. Compounds **35** and **37** have been characterized, *in vitro* and *in vivo*, displaying high potency and efficacy for PAR2 receptors in a variety of functional assays including cellular proliferation, calcium mobilization and PI hydrolysis assays, and both compounds stimulated internalization of PAR2 receptors [63]. The possible therapeutic utility of PAR2 agonists is controversial, though the identification of potent, selective, and metabolically stable small



35 (AC-55541)
pEC_{50} 6.7 ± 0.1

36 (AC-98170)
pEC_{50} 5.2 ± 0.2

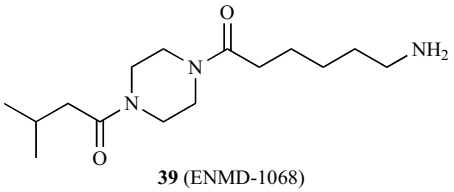
37 (AC-264613)
pEC_{50} 7.5 ± 0.1

38
pEC_{50} 7.6 ± 0.3

Fig. (6). Small-molecule compounds as PAR2 agonists. pEC_{50} were obtained using R-SAT.

molecule PAR2 ligands offers a number of new avenues for further exploration, including use in chronic studies, the development of radiolabeled probes for use in medical diagnostics and for binding studies, and development of antagonists.

PAR2 has represented a substantial challenge in terms of developing a high potency receptor-selective antagonist in fact, to date very few molecules have been described as showing an antagonist profile towards PAR2 receptor. The piperazine derivative **39** (ENMD-1068), Fig (**7**), based on the sequence LIGK-NH_2, that has shown the ability to block



39 (ENMD-1068)

Fig. (7). Structure of piperazine derivative PAR2 antagonist.

trypsin and PAR2-AP activation of the receptor, is a novel selective PAR2 antagonist that, despite its very low potency, has proved of use in *in vivo* studies [35]. Compound **39** (4 mg i.p.) in PAR2-null mice had no significant effect across 24 h compared with vehicle-treated PAR2-null mice, confirming **39** is not influencing inflammatory pathways other than those mediated *via* PAR2. In order to verify whether PAR2 represents a therapeutic target for the treatment of arthritis, an acute model of arthritis, obtained injecting 2% λ carrageenan and 4% kaolin in the knee joint, was used. The culmination of this study is the key finding that joint inflammation was dose dependently attenuated by prior i.p. administration of **39**, this effect being highly significant. The specificity of this compound was further confirmed by the observation that **39** (4 mg i.p.) had no effect on thrombin-mediated knee joint swelling over 48 h

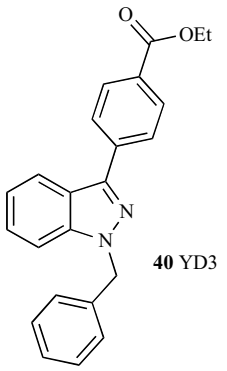
These results, in conjunction with the observation that disruption of the proteolytic activation of PAR2 using antiserum (B5) directed toward the receptor cleavage/activation site attenuated the inflammatory responses, have strongly contributed to assess the crucial involvement of PAR2 receptor in joint inflammation processes. These finding allows to identify PAR2 antagonists as potentially powerful anti-inflammatory agents for treatment of arthritis. That said, a high potent and selective PAR2 antagonist is yet to be reached and **39** could be considered only as a lead that requires further optimization.

PEPTIDE-MIMETIC PAR4 AGONISTS AND ANTAGONISTS

The complexity of PARs pharmacological profile is well exemplified by PAR4 actions firstly studied by means of human and murine PAR4-APs that have shown different effects on different tissues. GYPGQV-NH_2 and GYPGKF-NH_2, Fig. (**1**), are able to aggregate human and rodent platelets *via* PAR4, but are also able to cause either an endothelium-dependent nitric oxide mediated relaxant response in an endothelium-intact rat aorta (RA) preparation or a contractile response in a rat gastric longitudinal muscle (LM) preparation. Thus, several peptide agonists, based on the sequence of PAR4-APs, have been prepared in order to better characterize the tissue specificity and to assess the utility of such peptides for studies of PAR4 functions *in vivo*. Among these, AYPGKF-NH_2, obtained from AlaScan of murine PAR4-AP, resulted more potent than the native peptide. An early observation, regarding development of PAR1 antagonists, suggested that certain acylations at the N-terminus would give antagonist properties. In keeping with this statement, N-acylated derivatives, such as *trans*-cinnamoyl-YPGKF-NH_2 and *trans*-cinnamoyl-APGKF-NH_2 were prepared [64]. These two derivatives were able to inhibit platelet aggregation induced both by thrombin and PAR4-AP, AYPGKF-NH_2, but they acted as agonist on RA and LM preparations highlighting the differences existing between the platelet PAR4 receptor and the one(s) present in the RA and LM preparations.

SMALL-MOLECULE COMPOUNDS AS PAR4 ANTAGONISTS

In order to identify heterocyclic structures as PAR4 ligands, a screening of chemical compounds directed towards the evaluation of their ability to inhibit platelet aggregation allowed the identification of the newly synthetic indazole derivative **40** (YD-3, Fig. (**8**)) [65].



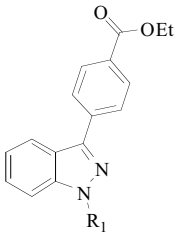
40 YD3

Fig. (8) Structure of compound **40** (YD-3).

Several studies have been performed demonstrating that compound **40** selectively inhibits rabbit platelet aggregation and phosphoinositide breakdown caused by thrombin; moreover it inhibits PAR4-dependent platelet activation and thromboxane formation aggregation [66, 67] and inhibits the thrombin-induced signal *via* Ras/extracellular-signal-regulated kinase (ERK), which critically influences cell proliferation in vascular smooth muscle cells *in vitro,* attenuating, also, the restenosis after balloon angioplasty *in vivo* [68]. In order to establish SAR of the lead **40**, a series of its derivatives have been synthesized and evaluated for selective anti-PAR4 activity [69]. The SAR study have demonstrated that both the 4"-ethoxycarbonyl and 1-benzyl groups on **40** contribute importantly to the selective anti-PAR4 activity. Any modification of the 4"-ethoxycarbonyl group decreased the activity supporting the evidence that this moiety is the major contributing functional group for the antiplatelet activity. The introduction of chloro, fluoro, or methoxy group onto the 1-benzyl group of **40** maintained its selective and potent anti-PAR4 activity, but led to considerable fluctuation in

potency. In particular, the substitution of chloro onto different sites of the 1-benzyl group led to 1-chlorobenzyl derivatives (**41-43**) with selective and potent anti-PAR4 activity (see Table (**3**)).

Table (3). The Inhibitory Effects of 40 and its Derivatives on Human Platelet Aggregation Induced by GYPGKF[a]



Compound	R_1	IC_{50} (μM)[b]
40	Benzyl	0.13 ± 0.02
41	2'-Chlorobenzyl	0.23 ± 0.02
42	3'-Chlorobenzyl	0.08 ± 0.01
43	4'-Chlorobenzyl	0.12 ± 0.01
44	2'-Fluorobenzyl	0.11 ± 0.00
45	3'-Fluorobenzyl	0.27 ± 0.03
46	4'-Fluorobenzyl	0.19 ± 0.01

[a]When the human platelet aggregation was induced by U46619, collagen, thrombin, and SFLLRN all the reported compounds gave IC_{50} values > 10 μM. [b]Human platelets were incubated with tested sample or 0.3% DMSO at 37 °C for 3 min, then U46619 (2 μM), collagen (10 μg/mL), thrombin (0.05 U/mL), SFLLRN (10 μM), or GYPGKF (1.5 mM) was added to trigger the aggregation.

Among them, 1-(4-chlorobenzyl) derivative **43** was found to be equally potent as **40**, whereas the 1-(3-chlorobenzyl) derivative **42**, with an IC_{50} of 0.08 μM, is more potent than the leading **40**. Similar substitution of fluoro atom onto the 1-benzyl group afforded 1-(fluorobenzyl) derivatives (**44-46**) with selective and potent anti-PAR4 activity.

PEPDUCINS AS PAR4 ANTAGONISTS

A novel approach to receptor inhibition has arose from the observation that N-palmitoylated peptides, termed pepducins, based on the intracellular loop 3 of certain GPCRs are able to cause activation and/or inhibition of G protein signalling only in the presence of the parent GPCR. In this configuration, the hydrophobic palmitoyl group is used to transport the peptide through the lipid bilayer targeting the intracellular surface of the GPCRs. Development of several cell penetrating peptides derived from the sequences of protease activated receptors led to good results when PAR4 has been considered. A relatively high potent PAR4 antagonist, named **P4pal10**, whose sequence is pal-SGRRYGHALR-NH_2, has been identified [70]. Although not completely selective for PAR4 and although the pepducins can have unusual non-PAR pharmacological actions, **P4pal10** has been tested both *in vivo* and *in vitro* demonstrating its ability to block PAR4. In particular, it has been verified that mouse platelets treated with **P4pal10** participate to a significantly lesser extent in thrombus initiation and growth when compared with non-treated platelets supporting the notion one of the most important mechanism of PAR pepducins action is the prevention of platelet–platelet (thrombus) interactions [71]. Moreover, the cardioprotective role of PAR4 antagonists has been evaluated by means of two structurally unrelated derivatives, such as **P4pal10** and *trans*-cinnamoyl-YPGKF-NH_2 [72]. **P4pal10** treatment before ischemia significantly decreased infarct size when given before, during, and after ischemia in the *in vivo* model. It has been also demonstrated that this cardioprotective effect were due to unmasked adenosine receptor signalling supporting the hypothesis of a coupling between thrombin receptors and adenosine receptors. Finally, the prior involvement of PAR4 receptors in inflammation processes has been recently revisited testing the effects of PAR4 inhibition in a model of systemic inflammation and disseminated intravascular coagulation (Shwartzman reaction) [40]. The authors have provided circumstantial evidence that the primary cellular target of the **P4pal10** may be neutrophils, rather than platelet or endothelial cells. The beneficial effect of PAR4 inhibition seems to be based on a reduced neutrophil infiltration into the site of the inflammation supporting the possible use of this approach in the treatment of systemic inflammation.

CONCLUSIONS

Protease activated receptors play a central role in orchestrating the interplay between coagulation, inflammation and fibroproliferation. A growing number of studies have assessed the crucial involvement of these receptors in inflammatory processes demonstrating that PAR activation can lead to either pro-inflammatory or protective effects. The tissue exposed to proteases is crucial as seen in the cases of PAR2 and PAR4 whose activation might generate opposite effects on different cellular types. Design and synthesis of PAR agonists and antagonists, based on the structures of the natural ligands, have been very challenging because of a consistent entropic factor that makes energetically preferred the anchored ligand binding mechanism with respect to a soluble ligand. Some progress has been made in identifying non-peptidic agonists or antagonists of PARs receptors such as the himbacine derived PAR1 antagonists [47-50], the piperazine derived PAR2 antagonist **39** [35] and the indazole derived PAR4 antagonists [65-69]. The structural optimization of these lead compounds, combined with more refined information on the three-dimensional structures of the targeted receptors, could allow to the identification of more selective and potent ligands.

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