Letters

Discovery of Potent and Selective Small-Molecule PAR-2 Agonists

Jimmi Gerner Seitzberg,[†] Anne Eeg Knapp,[†] Birgitte Winther Lund,[†] Sine Mandrup Bertozzi,[†] Erika A. Currier,[‡] Jian-Nong Ma,[‡] Vladimir Sherbukhin,[†] Ethan S. Burstein,[‡] and Roger Olsson*[,†]

ACADIA Pharmaceuticals AB, Medeon Science Park, Per Albin Hanssons väg 35, S-205 12 Malmö, Sweden, ACADIA Pharmaceuticals Inc., 3911 Sorrento Valley Boulevard, San Diego, California 92121

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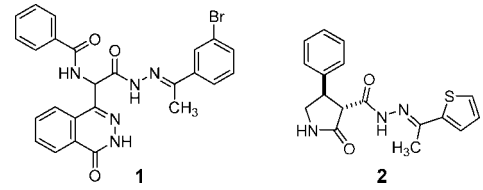
Abstract: Proteinase activated receptor-2 plays a crucial role in a wide variety of conditions with a strong inflammatory component. We present the discovery and characterization of two structurally different, potent, selective, and metabolically stable small-molecule PAR-2 agonists. These ligands may be useful as pharmacological tools for elucidating the complex physiological role of the PAR-2 receptors as well as for the development of PAR-2 antagonists.

Proteinase activated receptor-2 (PAR-2[a]) is considered to be an attractive target for drug discovery.[1] Physiologically, PAR-2 receptors are thought to fulfill a critical role in mediating nociceptive and inflammatory responses.[2,3] Therefore, drugs which target PAR-2 have the potential to treat a wide variety of disorders. Currently PAR-2 antagonists are of particular interest due to their potential for relieving inflammatory symptoms in rheumatoid arthritis. Paradoxically, PAR-2 agonists may also have therapeutic value. Although PAR-2 agonists would be expected to exacerbate most nociceptive and inflammatory processes, they have been suggested to have a protective role in certain settings. For example, PAR-2 agonists may have a therapeutic role as gastric cytoprotective agents or as mediators of airway smooth muscle relaxation.[4,5] The development of small-molecule ligands that selectively target PAR-2 will help the full assessment of PAR-2 receptors as therapeutic targets.

PAR-2 receptors belong to a subfamily of four G-protein coupled receptors (PAR-1, PAR-2, PAR-3, and PAR-4) and is widely expressed throughout the body including the CNS, cardiovascular, gastrointestinal, and pulmonary systems. The PARs are activated by tethered peptide ligands exposed by enzymatic proteolytic cleavage of the extracellular amino-terminus. PAR-1, PAR-3, and PAR-4 are activated by the protease thrombin, whereas PAR-2 is activated by trypsin and a variety of other proteases. In addition, PAR-1, PAR-2, and PAR-4 can be activated by soluble peptides derived from their tethered ligands (henceforth PAR activating peptides, or PAR APs).

Chart 1



Most research efforts aimed at elucidating the complex physiological roles of PAR-2 have relied on PAR-2 APs (e.g., SLIGRL), including recently more stable variants (e.g., 2-furoyl-LIGRLO-NH_2).[6] The susceptibility to proteolytic degradation of these peptides constitutes a major limitation for in vivo applications. To date, no nonpeptidic agonists of PAR-2 receptors have been reported,[7] which has hampered attempts to fully explore the function of the PAR-2 receptor. The discovery of metabolically stable small-molecule PAR-2 agonists would provide useful pharmacological tools for elucidating the complex physiological functions of PAR-2 receptors.

Herein we report the discovery and initial SAR of potent and selective nonpeptidic small-molecule PAR-2 agonists. A chemical library containing more than 250000 small-molecule drug-like compounds was screened for agonist activity at the human PAR-2 receptor using the cell-based functional assay R-SAT,[8] and a number of active compounds were identified. The hits could be divided into two different chemical classes exemplified by compound **1** (AC-55541),[9] displaying full agonism and nM activity at PAR-2 (pEC_{50} 6.7 and 81% efficacy), and the partial agonist **2** (AC-98170)[10] (pEC_{50} 5.2 and 30% efficacy) (Chart 1). The structure class that included the most potent and efficacious hit **1** was selected for further exploration.

A focused library around **1** was made by reacting a number of aromatic aldehydes or ketones with the parent hydrazide. From this library, an initial structure activity relationship (SAR) was established which 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 compound **1** were beneficial for the activity at PAR-2, any substituent in either the 2- or 4-position led to reduced activity. A variety of heteroaromatic moieties were also attached but all led to reduced activity compared to **1**.[11]

In addition to the good metabolic stability of **1** in human and rat microsomes (Cl_{int} h/rat 6/19 μL/min·mg), the compound also complies with the Lipinski rule-of-five. However, compound **1** had low solubility not only in phosphate buffer solution but also in other mediums such as organic solvents, which hampered the further use of this compound. Furthermore, the lack of reasonable building blocks made the synthesis of analogues of **1** quite demanding. These shortcomings led us to investigate the possibilities with the second structural class identified during the initial R-SAT screen. A hit-to-lead optimization effort of **2** was initiated by first taking advantage of the structural similarities between compounds **1** and **2**.

A molecular overlay of compounds **1** and **2** was performed using the flexible alignment procedure in MOE.[12] In addition to substitution in the aryl hydrazone part, the model suggested

* To whom correspondence should be addressed. Phone: +46406013400. Fax: +46406013401. E-mail: roger@acadia-pharm.com.
† ACADIA Pharmaceuticals AB.
‡ ACADIA Pharmaceuticals Inc.

[a] Abbreviations: PAR, proteinase activated receptor; PAR-AP, PAR-activating protein; SLIGRL, Ser-Leu-Ile-Gly-Arg-Leu-NH_2; 2-furoyl-LIGRLO-NH_2, 2-furoyl-Leu-Ile-Gly-Arg-Leu-Orn-NH_2; SFLLRN, Ser-Phe-Leu-Leu-Arg-Asn-NH_2; AYPGKF, Ala-Tyr-Pro-Gly-Lys-Phe-NH_2.

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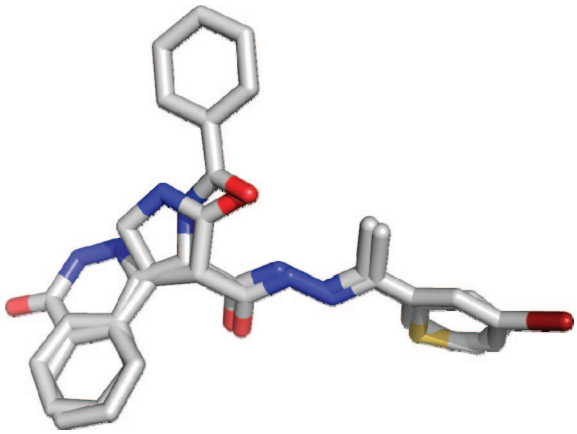
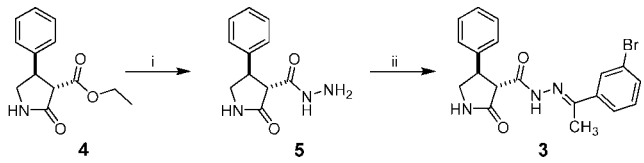


Figure 1. Molecular overlay of **1** and **2**.

Scheme 1. Synthesis of **3**[a]



[a] Reaction conditions: (i) H_2NNH_2, MW (120 °C, 20 min), 56%; (ii) 3-BrC_6H_4Ac, EtOH/AcOH 9/1, MW (120 °C, 600 s), 71%.

that substituents *ortho*- or *meta*- in the pyrrolidinone aromatic part should be tolerated as should substituents on the pyrrolidinone nitrogen (Figure 1).

This led to the synthesis of **3** (AC-264613),[10] which was easily prepared in two steps from commercially available ester **4** (Scheme 1).

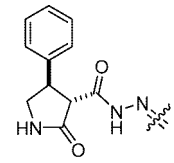
Compound **3** was highly potent at the PAR-2 receptor (entry 5, Table 1) and consolidated the new structure class that could be systematically modified to further explore the SAR and potentially incorporate desirable physicochemical properties.

The first set of analogues, prepared by the reaction of various aromatic ketones with hydrazide **5** (Scheme 1), were aimed at verifying that the SAR correlated with the results obtained previously with the **1** series (compounds **6**−**9**, Table 1). Analogues with substituents in the aromatic part of the pyrrolidinone were addressed by the synthesis of a focused library. The synthesis is outlined in Scheme 2, exemplified with the 3-bromo analogue **10**. The first step is a Michael addition between diethyl malonate and (*E*)-1-bromo-3-(2-nitrovinyl)benzene using sodium ethoxide as base. The crude Michael product **11** was reduced by Raney nickel using ammonium formate,[13] whereby the pyrrolidinone **12** was spontaneously formed as the thermodynamically more stable *trans*-isomer via an in situ cyclization.[14] The hydrazide **13** was formed by adding hydrazine to the ethyl ester **12** under microwave conditions. Finally, the hydrazide **13** was condensed with 3-bromoacetophenone, yielding the desired product **10**. The synthesis of N-methylated derivative **14** utilized a standard peptide coupling between acid **15** and hydrazone **16** (Scheme 3).

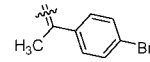
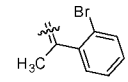
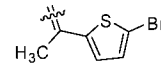
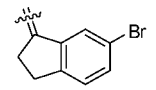
Substitution on the pyrrolidinone nitrogen was investigated and **19** was prepared by a three-step procedure in 26% yield (Scheme 4).[15]

The synthesized compounds were tested at human PAR-2 using R-SAT (Table 1). Initially, we explored the SAR around the aryl hydrazone part of the lead structure (entries 4−9). Substitution of the *meta*-position of the aryl was important for the activity, both the *para*- and *ortho*-substituted analogues **6** and **7** respectively showed more than 50-fold reduced activity compared to the *meta*-substituted compound **3** (compare entries

Table 1. In Vitro Activities of PAR-2 Agonists Using R-SAT[a]

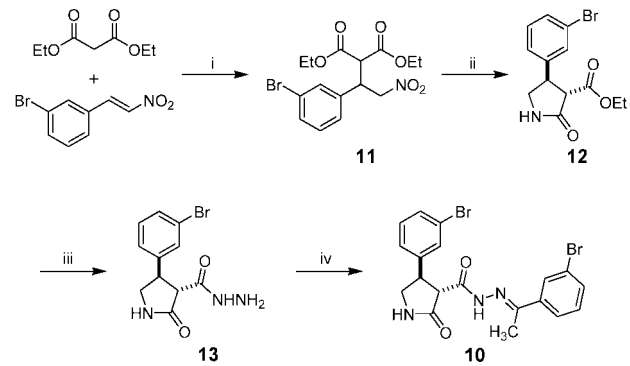


Compounds **6-9**

Entry	Compound	pEC_{50}	%Eff
1	SLIGRL-NH_2	5.0±0.3	100±0
2	2-f-LIGRLO-NH_2	7.6±0.1	89±11
3	**1**	6.7±0.1	81±4
4	**2**	5.2±0.2	30±9
5	**3**	7.5±0.1	93±15
6	 **6**	5.1±0.4	32±14
7	 **7**	5.0±0.5	11±4
8	 **8**	5.6±0.3	49±6
9	 **9**	6.9±0.2	62±7
10	**10**	7.6±0.3	82±6
11	**14**	5.2±0.7	35±8
12	**19**	7.3±0.2	72±12

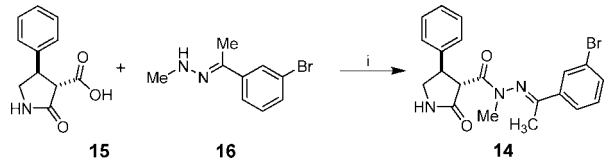
[a] It was not possible to separate the *E*- and *Z*-isomers and hence all structures were tested as mixtures of *E*- and *Z*-isomers on the imine part. See Supporting Information for details. R-SAT was performed as described in ref 8. Values represent the means ± SEM of three−nine independent experiments.

Scheme 2. Synthesis of **10**[a]



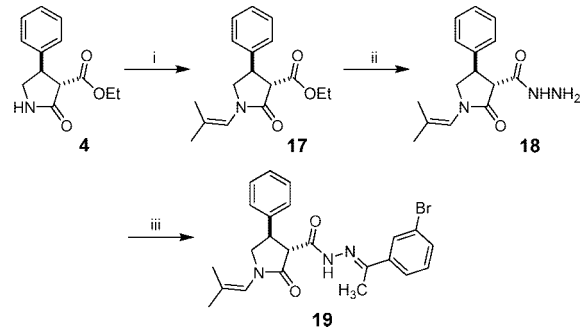
[a] Reaction conditions: (i) NaOEt, 0 °C, 2 h; (ii) (a) RaNi, HCO_2NH_4, EtOH, 70 °C, o.n. then rt, 3 days, (b) RaNi, H_2, rt, 24 h; (iii) $H_2NNH_2 \cdot H_2O$, MW 140 °C, 20 min, 16% (3 steps); (ii) 3-BrC_6H_4Ac, EtOH:AcOH 10:1, 50 °C, 16 h, 51%.

Scheme 3. Synthesis of **14**[a]



[a] Reaction conditions: (i) EDC, HOBT, THF, rt, 58%.

Scheme 4. Synthesis of **19**[a]



[a] Reaction conditions: (i) isobutyraldehyde, *p*-TsOH, MW 160 °C, 15 min, 62%; (ii) $H_2NNH_2 \cdot H_2O$, MW 120 °C, 20 min, 61%; (iii) 3-BrC_6H_4Ac, 10% AcOH in EtOH, 80 °C, 4 h, 70%.

5, 6, and 7). The 5-bromothiophene compound **8** gave about the same activity as the *para*-substituted compound **6** (entries 6 and 8). This was not surprising because the thiophene is regarded as a phenyl isoster and the 5-bromo substitution would more closely resemble a *para*-substituted phenyl than a *meta*-substituted one. The structurally more rigid dihydroindene **9** showed a slight decrease in activity compared to compound **3** (entries 5 and 9).

As indicated by the alignment of the hits **1** and **2**, substituents in the 2- and 3-position of the 4-phenyl of the pyrrolidinone were allowed. In line with the 3-bromo substituted compound **10**, several of the synthesized analogues were highly active, however, none of them displayed increased activity toward PAR-2 compared to compound **3**.[16] The N-methyl substituted compound **14** was inactive, indicating the importance of a hydrogen bond donor next to the carbonyl. Substituents on the pyrrolidinone nitrogen should be well tolerated according to the molecular alignment and, indeed, **19** was one of the more active compounds in the series.

Even though compound **3** was more active than hit **1** and had a low intrinsic clearance in vitro using liver microsomes (Cl_{int} for compound **3** is human/rat 9/37 μL/min·mg), the aqueous solubility of the compound was not improved. One contributing factor causing the low solubility of both compounds **1** and **3** may be the presence of hydrogen bond donor−acceptor pairs in the molecules. Hence, incorporating a substituent into either the hydrazide or the pyrrolidinone part of compound **3** would potentially increase solubility. However, these modifications did not improve the solubility of either **14** nor **19**. Providentially, the use of surfactants like Tween increased the solubility of compound **3** to a satisfying level (>2 mg/mL in 25% Tween), which makes this compound useful for further studies.

The enantiomers of **3** were separated using chiral HPLC and each enantiomer was tested for agonist activity at the PAR-2 receptor. It was found that the (+)-isomer was responsible for essentially all agonist activity (pEC_{50} 7.5 ± 0.0 and %Eff 91 ± 13 vs pEC_{50} < 5 and %Eff 10 ± 3 for the (−)-isomer).

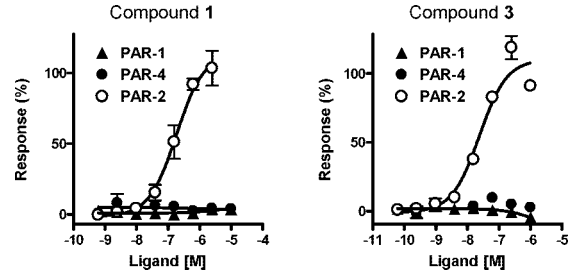


Figure 2. Receptor selectivity of PAR-2 agonists. Each compound was tested in concentration response experiments in R-SAT at PAR-1, PAR-4, and PAR-2 receptors. Activity of each receptor was verified with the PAR APs SFLLRN, AYPGKF, and SLIGRL for PAR-1, PAR-4, and PAR-2, respectively (not shown).

The activities of both compounds **3** and **1** were confirmed in phosphatidyl inositol (PI) hydrolysis and Ca^{2+} mobilization assays. The selectivity toward the other PAR subtypes was tested, and no activity of either **3** or **1** was observed at PAR-1, PAR-4 (Figure 2), or PAR-3.[17] Furthermore, neither compound had significant affinity in a profiling effort with more than 30 other targets implicated in nociception and inflammation. Importantly, the compounds show persistent and dose-dependent activity in vivo in paw edema and thermal hyperalgesia assays, characteristic of PAR-2 agonists.[17]

In conclusion, we have described the discovery and characterization of the first small-molecule nonpeptidic PAR-2 agonists. Both compounds **3** and **1** display significantly higher activities compared to the PAR-2 AP SLIGRL-NH_2. These compounds are highly selective for PAR-2 over the other PAR subtypes, they are metabolically stable and have persistent activity in vivo. Even though the possible therapeutic potential of PAR-2 agonists remains unclear, PAR-2 has been shown to play a crucial role in a wide variety of conditions with a strong inflammatory component. This indicates that PAR-2 could indeed be a novel target for drug development with both agonists and antagonists having therapeutic potential. The identification of potent, selective, and metabolically stable small-molecule PAR-2 agonists will be useful as pharmacological tools for elucidating the complex and at times contradictive physiological functions of the PAR-2 as well as for the development of PAR-2 antagonists.

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Supporting Information Available: Experimental details and characterization data for all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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(12) Molecular Operating Environment, Version 2004.03, Chemical Computing Group Inc. (http://www.chemcomp.com), 1010 Sherbrooke Street West, Suite 910, Montreal, Canada H3A 2R7. A number of conformations were generated for each of these two molecules using a stochastic search algorithm and the MMFF94 force field. These conformations were then superimposed in 3D to ensure the maximum overlap of their van der Waals volumes and pharmacophore features, like hydrogen bond donors and acceptors, aromatic rings, and lipophilic moieties. Each resulting alignment was assigned a score which quantified both feature overlap and internal strain energy.

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