Discovery and Optimization of a Novel Series of Thrombin Receptor (PAR-1) Antagonists: Potent, Selective Peptide Mimetics Based on Indole and Indazole Templates†

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Introduction. α-Thrombin is a key trypsin-like serine protease involved in coagulation and a variety of cellular actions, such as platelet aggregation, lymphocyte mitosis, monocyte chemotaxis, and endothelial cell proliferation.[1−4] Many of the effects of thrombin on cells are mediated by specific cell-surface receptors, which eluded identification for many years. However, in 1991, Coughlin and co-workers identified the first example of a thrombin receptor (protease-activated receptor-1, PAR-1), a member of the vast superfamily of seven-transmembrane, G-protein-coupled receptors.[5] Thrombin proteolytically cleaves the lengthy N-terminal extracellular domain of PAR-1 at the Arg-41/Ser-42 peptide bond to unveil a new N-terminus containing the recognition sequence SFLLRN, which appears to serve as a "tethered" peptide ligand. Interestingly, synthetic peptides containing this epitope exhibit full PAR-1 agonist activity,[6] and extensive structure−function data have been accumulated.[6−9] Three additional PARs, PAR-2,[10] PAR-3,[11] and PAR-4,[12] which are homologous to PAR-1, are now known, and each has its own agonist peptide motif.

PAR-1 mediates most of the cellular actions of thrombin, such as platelet aggregation, cell proliferation, inflammatory responses, and neurodegeneration. Thus, this receptor is an attractive drug discovery target for the possible treatment of various disorders such as thrombosis, restenosis, atherosclerosis, inflammation, cancer metastasis, and stroke.[2,13−15] Conceivably, a PAR-1 antagonist would be able to interfere with the cellular actions of thrombin without impacting on thrombin's role in coagulation and hemostasis. Additionally, a PAR-1 antagonist would provide a useful pharmacological tool for characterizing the physiological role of PAR-1, which has yet to be clearly delineated. Thus far, there has been a lack of specific and potent agents for blocking the actions of PAR-1 in vivo.

Given the tethered-ligand binding mechanism, an external ligand in competition would experience unfavorable energetics due to entropy considerations. Consequently, the identification of potent, small-molecule PAR-1 antagonists has been very challenging. The limited number of PAR-1 antagonists reported to date[7,16−22] have notable deficiencies such as weak potency, inability to block the action of thrombin on platelets consistently, mixed agonist/antagonist activity, and/or lack of PAR selectivity.[23] Indeed, our initial screening of nearly 200 000 library compounds for PAR-1 antagonist activity also did not generate viable leads. However, through a de novo design approach, we discovered a novel series of indole-based SFLLR peptide mimetics, containing many examples of potent, selective PAR-1 antagonists, which are able to block in vitro platelet aggregation induced by either SFLLRN-NH_2 or thrombin, but not by collagen, ADP, or the thromboxane mimetic U46619.[24] Herein, we report on the optimization of this series with the goal of conducting in vivo proof-of-principle studies to help establish the physiological role of PAR-1. We describe a second-generation indazole-based series, an archetype of which is **15** (RWJ-58259). Indazole **15**, a potent, selective PAR-1 antagonist with an improved in vivo cardiovascular safety profile, demonstrated in vivo antirestenotic activity in a rat balloon angioplasty model.

Synthetic Chemistry. The indole-based peptide mimetics **1**−**8** were prepared by either a convergent solution-phase method or an efficient 2-chlorotrityl resin-based solid-phase method.[24] Indazole-based antagonist **15** was prepared by a convergent solution-phase method as shown in Scheme 1. Thus, treatment of 6-nitroindole (**9**) with aqueous $NaNO_2$ under acidic conditions (pH = 1−2) afforded 3-indazolecarboxaldehyde **10**. Reductive amination of **10** with pyrrolidine/$NaBH(OAc)_3$ was followed by alkylation with 2,6-diCl-Bn-Br and nitro reduction with $Me_2NNH_2/FeCl_3$ to provide aminoindazole intermediate **12**. Coupling of *N*-α-Fmoc-*N*-γ-Boc-diaminobutyric acid (**13**) with benzylamine in the presence of DCC and HOBt was followed by deprotection of Fmoc group with diethylamine. The resulting intermediate was coupled with Fmoc-3,4-diF-Phe-OH using DIC/HOBt and treated with diethylamine to give dipeptide amine **14**. Urea formation between dipeptide amine **14** and 6-aminoindazole **12** in the presence of 4-nitrophenyl chloroformate was followed by deprotection of the Boc group with TFA to afford target compound **15**.

Optimization and Characterization of Bioactivity. Guided by structure−function data for SFLLRN-based agonist peptides,[6−9] in conjunction with extensive computer modeling, we proposed a "three-point model"[24] for peptide mimetic design. An indole template was among the selections of rigid molecular scaffolds for spatially displaying three key functional groups (amino, aryl, and guanidino) in a suitable pattern to obtain a worthwhile series of peptide mimetic PAR-1 antagonists. The indole template not only met the spatial

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† Abbreviations: PAR, protease-activated receptor; HMVEC, human microvascular endothelial cells; RASMC, rat aortic smooth muscle cells; PRP, platelet-rich plasma; GFP, gel-filtered platelet; ADP, adenosine diphosphate.

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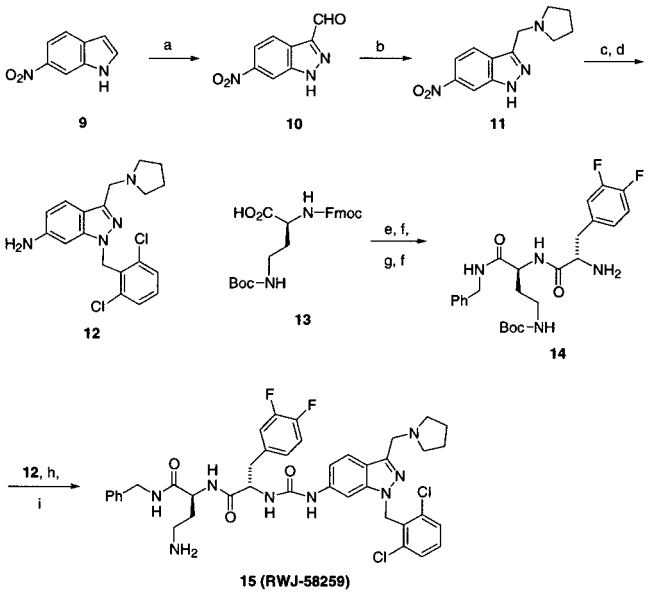
Scheme 1[a]

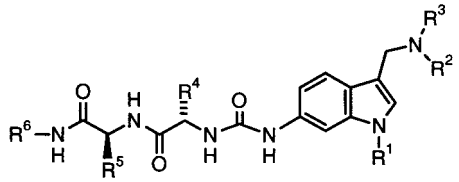


[a] Reagents and conditions (yield): (a) $NaNO_2$, 6 N HCl, 2.5 h; (b) pyrrolidine, $NaB(OAc)_3H$, $ClCH_2CH_2Cl$/DMF/HOAc (90:9:1), 1 h; (c) 2,6-dichlorobenzyl bromide, KOH, THF, 1 h; (d) $FeCl_3 \cdot 6H_2O$, charcoal powder, Me_2NNH_2, MeOH, reflux, 2 h (overall 24% **12** from **9**); (e) $BnNH_2$, DCC, HOBt, MeCN, 3 h; (f) Et_2NH, MeCN, 2 h; (g) Fmoc-3,4-diF-Phe-OH, DIC, HOBt, MeCN, 16 h (overall 65% **14** from **13**); (h) 4-nitrophenyl chloroformate, *i*-Pr_2NEt, CH_2Cl_2, −20 to 23 °C, 6 h; (i) CF_3CO_2H, CH_2Cl_2, 2 h (overall 26% **15** from **14**).

requirements but also offered favorable synthetic considerations for attachment of the desired substituents. After synthesizing many indole derivatives, we identified indole-based peptide mimetic **1** (Table 1, synthesized in nine steps in solution phase[24]) as an early lead PAR-1 antagonist. Compound **1** exhibited high PAR-1 affinity (IC_{50} = 0.7 μM) in a radioligand binding assay, involving competitive binding of [^{3}H]-*S*-(*p*-F-Phe)-homoarginine-L-homoarginine-KY-NH_2 to the PAR-1 on membranes from CHRF-288-11 cells,[24] and potent inhibition against platelet aggregation induced by SFLLRN-NH_2 (IC_{50} = 0.30 μM). However, **1** was relatively weak against platelet aggregation induced by thrombin[24] (IC_{50} = 13.3 μM) and not selective relative to collagen (IC_{50} = 12.7 μM). To explore the SAR and optimize the potency, rapidly, we developed efficient secondary amide resin-[25] and 2-chlorotrityl resin-based,[24] parallel solid-phase methods to synthesize diverse analogues, where we could alter sites around the indole scaffold (R^1, R^2, R^3, R^4, R^5, and R^6), as shown in the general structure in Table 1. Altogether, nearly 1200 related compounds have been prepared and tested. This effort led to a good understanding of the SAR and a significant improvement in potency and PAR-1 selectivity. Representative compounds with in vitro biological data are shown in Tables 1 and 2.

Replacement of Phe (R^4) in **1** with 4-MeO-Phe (**2**) gave a 6-fold increase of potency against thrombin-induced gel-filtered platelet (GFP) aggregation (IC_{50} = 2.3 μM) and much better selectivity over collagen and U46619. Evaluation of **2** in nonplatelet functional assays (calcium signaling and proliferation of vascular cells)[24] indicated that its potency in inhibiting the effects of thrombin was just moderate, with IC_{50} values in the HMVEC and RASMC calcium mobilization assays of 0.54 and 7.4 μM,

Table 1. Indole-Based Peptide Mimetics as PAR-1 Antagonists[a]



compd	R^1	R^2R^3N	R^4	R^5	R^6
1	4-F-Bn	$(CH_2)_4N$	Phe	Arg	Bn
2	4-F-Bn	$(CH_2)_4N$	4-MeO-Phe	Arg	Bn
3	4-F-Bn	*c*-C_5H_9NH[b]	4-MeO-Phe	Arg	Bn
4	4-CF_3O-Bn	$(CH_2)_4N$	4-MeO-Phe	Arg	Bn
5	2,6-diCl-Bn	$(CH_2)_4N$	4-MeO-Phe	Arg	Bn
6	2,6-diCl-Bn	$(CH_2)_4N$	3,4-diF-Phe	Arg	Bn
7	2,6-diCl-Bn	$(CH_2)_4N$	3,4-diF-Phe	Arg	4-Py-CH_2[c]
8	2,6-diCl-Bn	$(CH_2)_4N$	3,4-diF-Phe	Dbu[d]	Bn

[a] R^4 and R^5 denote side chains of amino acids. [b] *c*-C_5H_9 = cyclopentyl. [c] Py = pyridyl. [d] Dbu = 2,4-diaminobutyric acid.

respectively, and in the RASMC proliferation assay of more than 30 μM. Attempts to substitute the pyrrolidine (R^2R^3N) in **2** with cyclopentylamine (**3**) or the F substituent of R^1 in **2** with OCF_3 (**4**) resulted in a significant decrease of potency in platelet aggregation. Systematic study of substituents around the benzyl group at the R^1 position surfaced ca. 3-fold more potent analogue **5**, which has 2,6-diCl-Bn at the R^1 position. A further increase of potency was achieved by replacing the 4-MeO-Phe in **5** with 3,4-diF-Phe (**6**), which potently inhibited platelet aggregation induced by thrombin (IC_{50} = 0.57 μM) or SFLLRN-NH_2 (IC_{50} = 0.18 μM), while being quite selective relative to collagen and U46619 (both IC_{50} > 50 μM). Also, **6** exhibited high affinity for PAR-1 (IC_{50} = 0.82 μM) in the binding assay and showed a significant improvement in potency over **2** in nonplatelet functional assays (Table 2). Analogue **7**, with the benzyl group of R^6 in **6** replaced by 4-pyridylmethyl, bound to PAR-1 with surprisingly high affinity (IC_{50} = 0.04 μM), although **7** had fairly standard potency in the GFP aggregation assays (Table 2). The reason for this dichotomy in potency for **7**, between the PAR-1 binding and functional activity, is not yet understood. However, other researchers have also observed inconsistencies between receptor binding and platelet aggregation inhibition for PAR-1 antagonists.[23]

We sought to evaluate **6** in a model of ex vivo platelet aggregation. Guinea pigs were chosen for study because their platelets have a similar thrombin receptor profile to human platelets, and it was crucial to have a small-animal model to conserve test compound. Unfortunately, iv administration of **6** to anesthetized guinea pigs caused severe hypotension at a dose of 3 mg/kg, which was subsequently shown to be independent of PAR-1 activity. Followup cardiovascular assessment of selected compounds in the series in an anesthetized guinea pig hemodynamic model suggested that the guanidine residue in **6** might be responsible for the undesirable cardiovascular effects. Therefore, an effort was made to replace the guanidine moiety, originally believed to be important for PAR-1 activity, with other functionalities. We found success by replacing the Arg side chain of **6** with a 2-aminoethyl side chain, which led to the potent analogue **8** (RWJ-56110; Table 2). Fortunately, **8** showed a significantly improved cardiovascular hemodynamic

Table 2. In Vitro Biological Data of PAR-1 Antagonists[a]

	GFP aggregation[b]					HMVEC[d]	RASMC[e]	RASMC[f]
compd	thrombin	SFLLRN-NH_2	collagen	U46619	recpt binding[c]	Ca^{2+} flux	Ca^{2+} flux	prolif
1	13.3 ± 6.8 (3)	0.30 ± 0.18 (2)	12.7 ± 2.6 (3)	NT	0.7 (1)	NT	NT	NT
2	2.32 ± 0.11 (184)	0.27 ± 0.01 (172)	IA	IA	2.2 ± 0.5 (23)	0.54 ± 0.18 (2)	7.8 ± 2.4 (2)	>30
3	17.8 ± 3.6 (2)	2.2 ± 1.0 (4)	41.4 ± 12.7 (3)	49.1 (1)	10.7 (1)	NT	NT	NT
4	18.2 ± 0.9 (3)	3.20 ± 0.66 (2)	24.2 ± 8.4 (3)	25.5 ± 7.9 (3)	2.4 (1)	NT	NT	NT
5	0.90 ± 0.34 (3)	0.30 ± 0.09 (3)	46.3 ± 28.6 (3)	IA	1.3 ± 0.00 (2)	0.33 (1)	0.93 (1)	7.3 (1)
6	0.57 ± 0.21 (8)	0.18 ± 0.21 (10)	IA	IA	0.82 ± 0.34 (4)	0.09 ± 0.04 (3)	0.13 ± 0.1 (3)	2.4 ± 0.7 (3)
7	1.06 ± 0.52 (3)	0.36 ± 0.05 (2)	IA	13.3 (1)	0.04 ± 0.01 (2)	0.17 ± 0.01 (4)	0.21 ± 0.07 (3)	6.5 (1)
8	0.34 ± 0.04 (13)	0.16 ± 0.05 (15)	IA	IA	0.44 ± 0.21 (6)	0.13 ± 0.07 (3)	0.12 ± 0.02 (2)	3.5 ± 0.5 (4)
15	0.37 ± 0.07 (12)	0.11 ± 0.01 (2)	IA	IA	0.15 ± 0.05 (3)	NT	0.07 ± 0.01 (4)	2.3 ± 0.0 (2)

[a] Results are expressed as mean ± SEM (IC_{50}, μM) with the number of experiments (N) in parentheses. IA denotes inactivity @ 50 μM of test compound. NT denotes not tested. [b]Concentrations of agonists for aggregation studies: α-thrombin, 0.15 nM; SFLLRN-NH_2, 2 μM; collagen, 3 μg/mL; U46619, 0.3 μM. [c]Thrombin receptor (PAR-1) binding assay; ligand: [^{3}H]-*S*-(*p*-F-Phe)-homoarginine-L-homoarginine-KY-NH_2, 10 nM (K_d = 15 nM). [d]HMVEC calcium mobilization assay; 2.0 nM α-thrombin. [e]RASMC calcium mobilization assay; 2.0 nM α-thrombin. [f]RASMC proliferation assay; 0.8 nM α-thrombin.

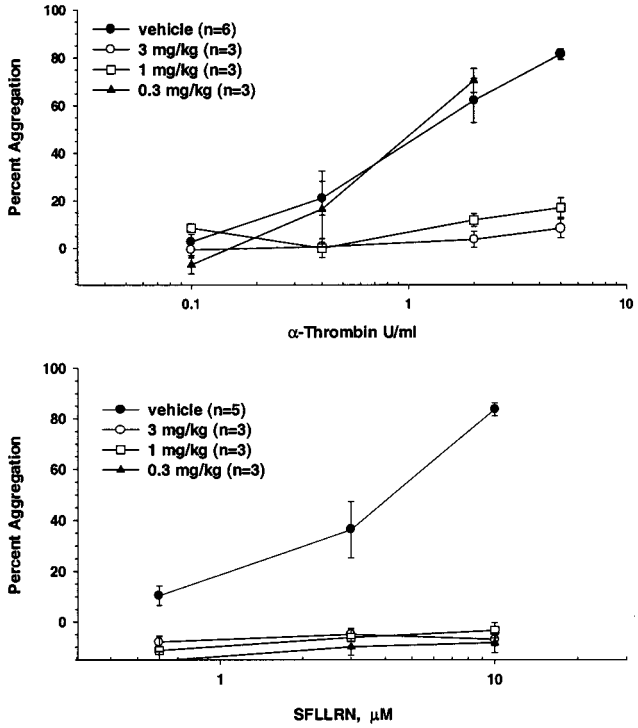


Figure 1. Inhibition of ex vivo platelet aggregation in guinea pigs by **8**.

profile over **6**, with only minimal hypotensive effects up to 3 mg/kg, iv, in guinea pigs.

In an ex vivo protocol, **8** was infused iv into anesthetized guinea pigs at 0.3, 1, or 3 mg/kg over 5 min. Blood was drawn 5 min after the end of the infusion to determine the extent of thrombin- or SFLLRN-NH_2-induced aggregation of PRP by using a microplate method. We observed a dose-dependent inhibition of ex vivo PRP aggregation induced by both agonists (Figure 1). Thrombin-induced aggregation was not significantly inhibited by the lowest dose evaluated, 0.3 mg/kg, but was substantially inhibited by higher concentrations of **8**. Furthermore, the inhibition was sustained at high thrombin concentrations, up to 5 U/mL. SFLLRN-NH_2-induced aggregation was inhibited at all doses tested. These results constitute an ex vivo proof-of-concept for a PAR-1 receptor antagonist.

At a dose of **8** of 7 mg/kg, iv, in guinea pigs, we observed moderate hypotensive responses. In vivo evaluation of diverse analogues suggested that the size of the substituent at position 2 of the indole nucleus partly contributed to these side effects. To improve the therapeutic index, we replaced the indole with an indazole, which has the smallest possible group at position 2, a lone electron pair. This modification resulted in the identification of a second-generation, indazole-based series of potent and selective PAR-1 antagonists, an archetype of which is **15** (RWJ-58259; synthesis shown in Scheme 1). Indazole **15** turned out to be an improved lead in both in vitro and in vivo protocols. Compared with indole **8**, indazole **15** exhibited improved potency in the binding and nonplatelet functional assays (Table 2), while being equipotent and PAR-1-selective in the platelet aggregation assays. Significantly, iv administration of **15** to anesthetized guinea pigs showed a better hemodynamic profile relative to **8**, with no significant effects up to 7 mg/kg. In the guinea pig model of ex vivo platelet aggregation, **15** also showed improved efficacy over **8**; it was able to inhibit completely ex vivo platelet aggregation induced by 2 U/mL thrombin at a dose as low as 0.3 mg/kg, iv.[26]

With an improved cardiovascular safety profile and improved efficacy, indazole **15** was a good candidate to assess the therapeutic potential of a PAR-1 antagonist in disease models. Since thrombin is a powerful stimulator of platelet aggregation and degranulation, as well as a significant mediator of platelet recruitment during arterial thrombus formation, there is keen interest in the potential for antithrombotic action from antagonism of PAR-1. However, little progress has been achieved in this area primarily because of the lack of a suitable pharmacological agent to block PAR-1 function in vivo. We examined **15** in two standard thrombosis models in guinea pigs: the arteriovenous (AV) shunt assay (monitoring thrombus weight) and the Rose Bengal intravascular photoactivation assay (monitoring time to occlusion).[26] At an iv total dose of 10 mg/kg, **15** failed to inhibit thrombus formation in these models. However, a modest antithrombotic effect (thrombus weight reduction from 35 ± 2 to 24 ± 4 mg) was observed in the AV shunt model when **15** was delivered locally, i.e., directly into the shunt. These results are consistent with the dual-PAR activation system, PAR-1 and PAR-4, that exists on guinea pig platelets.[24,26]

Unlike human or guinea pig platelets, which possess PAR-1 and PAR-4, human or rat vascular smooth muscle cells express only PAR-1. Thus, we examined the ability of **15** to block thrombin-induced RASMC calcium mobilization and proliferation (Table 2). In

these assays, **15** was completely effective, even at high thrombin concentrations (up to 200 nM; results not shown), which suggests the inherent potential of **15** to treat the vascular diseases. Since thrombin has been implicated in the proliferative and inflammatory events associated with restenosis, we evaluated **15** in a vascular restenosis model involving balloon angioplasty in rats. Significant reduction of neointimal thickness (45 $\pm$ 5 μm in treated animals vs 77 $\pm$ 5 μm in controls, $p < 0.05$) was observed by administering **15** perivascularly, basically, by implanting 10 mg of **15** in a hydrogel formulation.[26,27] This favorable outcome clearly demonstrates an important role for PAR-1 in vascular injury.

Conclusion. We have discovered a novel series of indole-based SFLLR peptide mimetics as potent, selective PAR-1 antagonists and optimized the series for in vivo studies by altering the guanidine-containing side chain and introducing an indazole template. Indazole-based PAR-1 antagonist **15** has improved in vivo efficacy and cardiovascular safety and served as a pharmacological tool for assessing the therapeutic potential of a PAR-1 antagonist in disease models. Although **15** was not particularly effective in two guinea pig thrombosis models, probably because of the presence of PAR-4 on guinea pig platelets, it was effective in reducing restenosis in a rat balloon angioplasty model. Consequently, a selective PAR-1 antagonist could be beneficial for treating restenosis attendant to arterial injury, such as that following balloon angioplasty.

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

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