

# Potent Non-Peptide Thrombin Receptor Antagonists

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**Abstract:** Protease activated receptor-1 (PAR-1), also known as thrombin receptor, is present in a variety of cell types such as platelets and endothelial cells. PAR-1 is proteolytically activated by thrombin by cleavage at its extracellular domain, unmasking a new amino terminus, which internally binds to the proximal receptor, eliciting cellular activation. Inhibition of the cellular activation by thrombin is a potentially promising therapeutic approach for the treatment of thrombotic and vascular proliferative disorders such as atherosclerosis and restenosis. Reported herein is the pharmacology of potent, low molecular weight thrombin receptor antagonists from pyrroloquinazoline, benzimidazole, and himbacine series. In the radioligand binding assay, these compounds inhibited PAR-1 in a competitive manner. They also inhibited thrombin and agonist peptide induced human platelet aggregation in a dose-dependent manner. Additionally, these compounds showed dose-dependent inhibition of agonist-induced cytosolic  $Ca^{+2}$  transients and thymidine incorporation in human coronary artery smooth muscle cells (hCASMC). The most potent compound among these antagonists showed a  $K_i$  of 12 nM in the radioligand binding assay and an  $IC_{50}$  of 70 nM in the platelet aggregation inhibition assay.

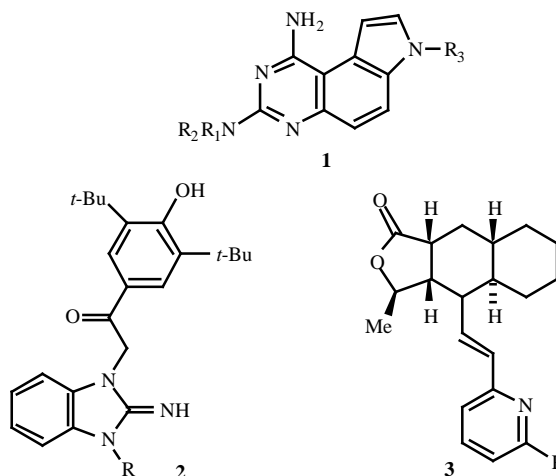
**Keywords:** PAR-1, PAR-1 antagonist, thrombin receptor antagonist, PAR-1 inhibitor, protease activated receptor, pyrroloquinazoline, benzimidazole, himbacine, TRAP.

## INTRODUCTION

In addition to its pivotal role in hemostasis [1], thrombin mediates potent proliferative and proinflammatory processes in a variety of cell types by direct cellular activation [2]. Cellular actions of thrombin are mediated by the activation of specific cell surface receptors known as protease activated receptors (PAR) that comprise a small family of G-protein coupled receptors [3]. The prototype of this new class of receptors, known as protease activated receptor-1 (PAR-1) or the thrombin receptor, is present in a variety of human cell types including platelets, fibroblasts, endothelial cells, and cardiac myocytes [4]. The potential pathophysiological role of the thrombin receptor in thrombosis, atherosclerosis, and restenosis has been well recognized. As such, a thrombin receptor antagonist may have considerable therapeutic utility in the treatment of these disorders. Since a thrombin receptor antagonist is specific for the cellular actions of thrombin and does not interfere with the coagulation cascade, such agents are likely to confer added safety margin with regard to hemorrhagic side effects which is a complicating factor in the currently available antithrombotic treatment [5]. Several peptide agonists and antagonists of PAR-1 based on the amino acid sequence of the "tethered ligand" have been reported [6]. Recently there have been reports of nonpeptide PAR-1 antagonists from our own laboratory [7,8] as well as other laboratories [9].

Herein we wish to review the pharmacology of three different classes of low molecular weight thrombin receptor antagonists. These are pyrroloquinazolines (1), benzi-

midazoles (2), and a novel class of thrombin receptor antagonists represented by structure 3, based on the natural product himbacine (Fig. 1). These compounds were initially screened in the radioligand binding assay using [ $^3H$ ]ha-TRAP [10]. Promising compounds were further characterized by functional assays such as platelet aggregation assay, cytosolic  $Ca^{+2}$  measurement assay, and cell proliferation assay [7e].



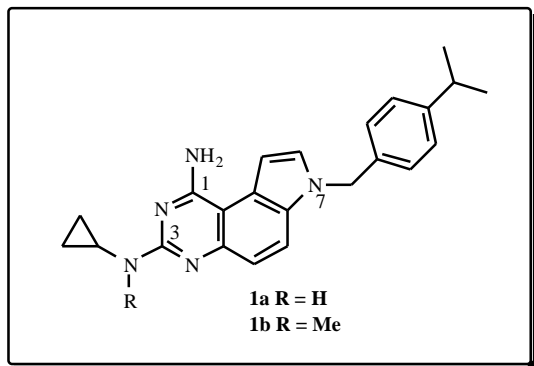
**Fig. (1).** Low-molecular-weight thrombin receptor antagonists: (1) pyrroloquinazoline derivatives, (2) benzimidazole derivatives, (3) himbacine derivatives.

## PYRROLOQUINAZOLINE-DERIVED THROMBIN RECEPTOR ANTAGONISTS

The synthesis and SAR of this class of compounds have been reported [7b]. The amino group at C<sub>1</sub> and the *p*-

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(isopropyl)benzyl group at N<sub>7</sub> were essential for PAR-1 antagonism. Among the substituents at C<sub>3</sub>, *N*-cyclopropylamino derivatives **1a** and **1b** were the most active. In the radioligand binding assay using [<sup>3</sup>H]-ha-TRAP, **1a** and **1b** gave IC<sub>50</sub> values of 70 nM (K<sub>i</sub> = 35 nM) and 45 nM (K<sub>i</sub> = 22 nM), respectively. Analysis of saturation binding of [<sup>3</sup>H]ha-TRAP in the presence and absence of compound **1a** indicated that this compound is a competitive inhibitor of PAR-1.



Compounds **1a** and **1b** blocked platelet aggregation induced by PAR-1 selective agonist ha-TRAP in a concentration-dependent fashion, with IC<sub>50</sub> values of 300 and 150 nM, respectively. The inhibition of platelet aggregation by compounds **1a** and **1b** were selective, as evidenced by the fact that at 10 μM they had no effect on aggregation induced by 100 μM ADP or 5 mM collagen. Both compounds also inhibited aggregation induced by -thrombin with IC<sub>50</sub> values of 3000 and 700 nM, respectively. In contrast to the sustained inhibition of ha-TRAP-induced aggregation, the inhibition of thrombin induced aggregation was transient and the observed delay in aggregation was

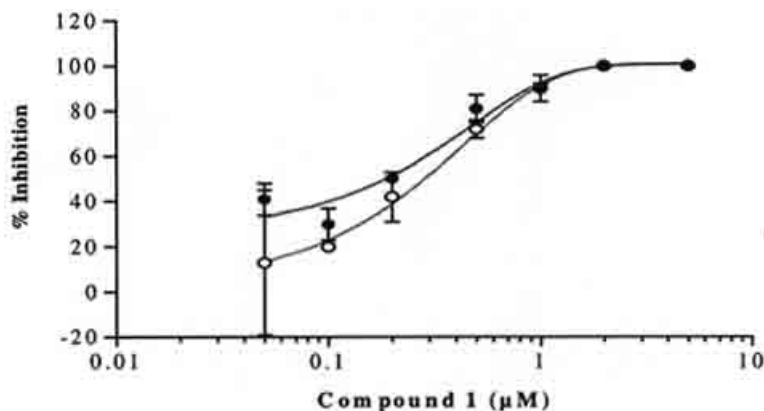
dependent on the concentration of thrombin used. At 0.5 nM thrombin, full aggregation was delayed by several minutes, whereas at 10 nM thrombin, no significant delay was seen. These compounds did not inhibit aggregation induced by PAR-4 tethered ligand peptides, nor did they have any effect on platelet aggregation induced by -thrombin. Binding of these drugs to platelet was reversible, and full reversal of inhibition required platelets to be washed free of drug for 20 min. These compounds had no agonist activity at concentrations as high as 3 μM, nor did they inhibit the catalytic activity of thrombin.

Compound **1a** inhibited calcium transients induced by thrombin (3 nM) and the peptide agonist TFLLRNPNDK-NH<sub>2</sub> (30 μM) with K<sub>i</sub> values of 82 and 55 nM, respectively. In contrast to platelets, where the inhibition of thrombin-induced effect was transient, inhibition of the calcium transients in hCASM C was sustained over the time course of the assay (Fig. 2).

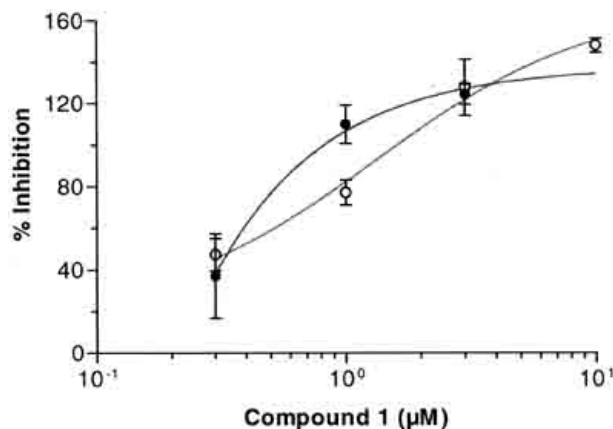
In the smooth muscle cell proliferation assay, pyrroloquinazoline derivative **1a** completely inhibited thrombin and TFLLRNPNDK-NH<sub>2</sub>-stimulated [<sup>3</sup>H]thymidine incorporation, with apparent K<sub>i</sub> values of 88 and 32 nM, respectively. As observed with calcium flux experiments, inhibition of thrombin-induced thymidine incorporation was sustained throughout the incubation period of the assay (Fig. 3).

## BENZIMIDAZOLE DERIVATIVES

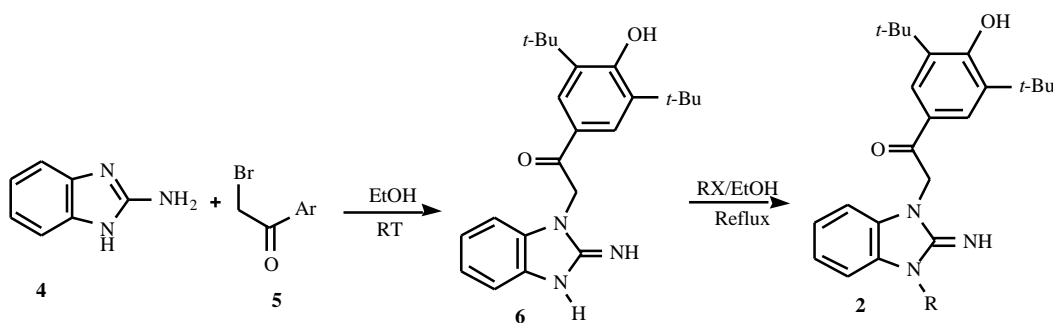
Benzimidazole derivatives represented by structure 2 were readily prepared according to the literature procedure as shown in Scheme 2 [7a, 12]. Sequential *N*-1 and *N*-3 alkylation of 2-aminobenzimidazole (**4**) was carried out using commercially available phenacyl bromides and alkyl halides (Scheme 2). In the first step, dialkylation was minimized by



**Fig. (2).** Inhibition of thrombin- and TFLLRNPNDK-NH<sub>2</sub>-stimulated calcium transients in hCASM C by **1a**. Compound **1a** was added 30 min before the addition of thrombin (3 nM) and TFLLRNPNDK-NH<sub>2</sub> (30 μM). The final concentration of vehicle (DMSO) was 0.2%. Key: (●) 3 nM thrombin; and (○) 30 μM TFLLRNPNDK-NH<sub>2</sub>. The K<sub>i</sub> value was calculated using the following Cheng-Prusoff equation,  $K_i = IC_{50}/(1+[A]/[EC_{50}])$ , where IC<sub>50</sub> is the antagonist concentration for inhibition of an agonist effect by 50%, [A] is the concentration of the agonist used, and EC<sub>50</sub> is the agonist concentration for half-maximal stimulation. Each value is the mean ± SEM for *n* = 4. All points (% inhibition) with the exception of one point (0.03 μM of **1a** against TFLLRNPNDK-NH<sub>2</sub>) were significantly different from control (no **1a**) (*P* < 0.05). These data are representative of three separate experiments with essentially similar results.



**Fig. (3).** Inhibition of thrombin- and TFLLRNPNDK-NH<sub>2</sub>-stimulated [<sup>3</sup>H]thymidine incorporation in hCASMC by **1a**. Compound **1a** was added 30 min before addition of PAR-1 agonists. The final concentration of vehicle (DMSO) was 0.2%. Key: (●) 3 nM Thrombin; and (○) 10 μM TFLLRNPNDK-NH<sub>2</sub>. Amastatin (50 μM) was present in the incubation medium when TFLLRNPNDK-NH<sub>2</sub> was tested. Each point and bar is the mean ± SEM for *n* = 4.



**Scheme 2.**

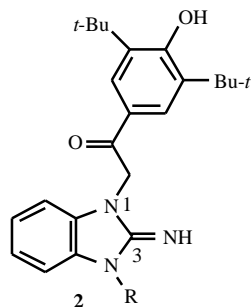
using the alkylating agent as the limiting reagent. The second alkylation often required refluxing conditions and the product could be easily isolated by crystallization from the reaction mixture. IC<sub>50</sub> determinations were carried out on PAR-1 receptors on human platelets as described above. The structure–activity relationship data are presented in Table 1.

The monosubstituted benzimidazole derivative **2a** showed an IC<sub>50</sub> of 1500 nM. Systematic variation of the *N*-3 substitution pattern revealed that a lower alkyl or benzyl group yielded optimum activity. For example, the *N*-3-butyl derivative **2i** gave an IC<sub>50</sub> value of 359 nM whereas the *N*-3-methyl derivative (**2b**) and *N*-3-heptyl derivative (**2j**) were less active. Compound **2c** bearing 2-(1-piperidinyl)ethyl group was somewhat less potent (IC<sub>50</sub> = 2870 nM) whereas the corresponding morpholine derivative **2f** was quite potent (IC<sub>50</sub> = 98 nM). Compound **2g**, bearing *N*-3-benzyl substituent, gave an IC<sub>50</sub> of 65 nM. Phenyl substituent effect on the *N*-3-benzyl group was also studied (**2d–2e** and **2h**). Among the various *N*-3-benzyl derivatives examined, compound **2h**, bearing a *p*-tolyl group, showed the best activity (IC<sub>50</sub> = 33 nM).

Several compounds in the benzimidazole series were also tested in functional platelet aggregation assay employing ha-TRAP and thrombin to induce aggregation. The results for compounds **2f–2i** are shown in Table 2. These compounds were effective inhibitors of ha-TRAP-induced platelet aggregation, although the IC<sub>50</sub> values for blocking the aggregation were consistently higher than those observed in the binding assay. The compounds also inhibited thrombin-induced platelet aggregation. The rank order of potency paralleled that for ha-TRAP inhibition, but higher concentrations of the compounds were needed to produce inhibition.

## HIMBACINE ANALOGS

Himbacine (**7**) is a tetracyclic piperidine alkaloid isolated from the bark of Australian magnolia trees [13]. The strong antimuscarinic activity that himbacine was reported to possess drew our attention to the SAR exploration of this class of compounds, as part of our discovery efforts addressed to Alzheimer's disease [14]. This effort eventually culminated in a total synthesis of himbacine and the

**Table 1. Structure–Activity Relationship of Substituted Benzimidazoles (2)**

Entry	R	PAR-1 IC <sub>50</sub> (nM)	Entry	R	PAR-1 IC <sub>50</sub> (nM)
2a	H	1500	2f	 Benzyl	98
2b	Me	900	2g	Benzyl	65
2c		2870	2h		33
2d		734	2i	<i>n</i> -C <sub>4</sub> H <sub>9</sub>	359
2e		1320	2j	<i>n</i> -C <sub>7</sub> H <sub>15</sub>	964

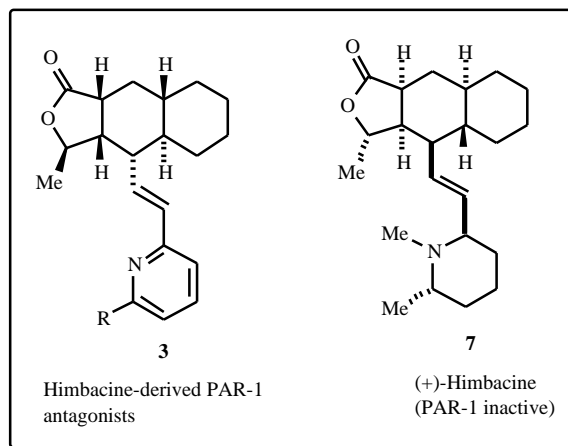
synthesis of a number of its analogs, from which emerged the current himbacine-based PAR-1 lead.

**Table 2. Inhibition of ha-TRAP and Thrombin-Induced Human Platelet Aggregation by Benzimidazole Derivatives (2)**

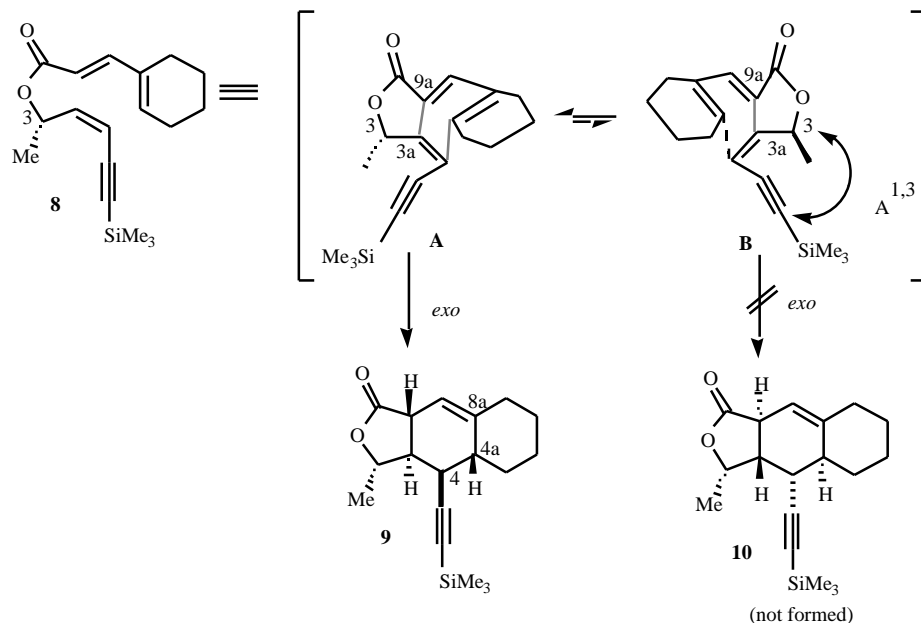
Entry	PAR-1 IC <sub>50</sub> (nM)	Platelet Aggregation IC <sub>50</sub> (nM) <sup>10</sup>	
		ha-TRAP	Thrombin
2f	98	2000	>10,000
2g	65	265	600
2h	33	575	1500
2i	359	825	10,000

Himbacine *per se* is devoid of any PAR-1 activity. Himbacine-based PAR-1 antagonists have several unique structural features that distinguish themselves from the natural product. First, the PAR-1 antagonists have a

substituted vinyl pyridine in place of the 2,6-disubstituted piperidine moiety that is present in the natural product. Secondly, as described below, the *ent*-himbacine absolute stereochemistry of the tricyclic ring system is preferred. Another interesting trait of himbacine-based PAR-1 antagonists is that they are totally devoid of any muscarinic activity.



The synthesis of himbacine-based PAR-1 antagonists employs a highly diastereoselective intramolecular Diels-Alder reaction (IMDA) of trienyl-yne **8** as the key step for the construction of the tricyclic ring system [13]. Several points are worth noting regarding this approach. The facial selectivity of the C<sub>3a</sub>-C<sub>9a</sub> bond formation during the IMDA is governed by the preferred conformation **A** of the IMDA precursor **8** which entails less A [1,3] strain than the alternative conformation **B** (Scheme 3). Once the relative configuration between the C<sub>3</sub> and C<sub>3a</sub> is established preferentially, the relative configurations at the remaining stereogenic centers follow the desired pattern due to the topology of the Diels-Alder transition state and the double bond geometry of the dienophile. For example, the relative configuration at C<sub>9a</sub> is established *trans* with respect to C<sub>3a</sub> due to the *exo*-selective nature of the IMDA. Although this transition state temporarily generates the undesired relative configuration at C<sub>9a</sub>, it helps establish the correct relative configuration at C<sub>4a</sub> by virtue of the concerted ring closure inherent to the Diels-Alder process. The stereogenic center at C<sub>9a</sub> can be readily epimerized since the resultant *cis*-lactone is thermodynamically more stable. The relative configuration at C<sub>4</sub> is the outcome of the *cis*-geometry of the dienophile. Finally, we have demonstrated in the context of the total synthesis of (+)-himbacine that the C<sub>8a</sub>-C<sub>9</sub> double bond of the *cis*-lactone can be stereoselectively reduced from the  $\alpha$ -face, thereby establishing the required relative configuration at C<sub>8a</sub>. By employing optically pure 3-butyn-2-ol (Scheme 4), this ensemble allows transmission of the C<sub>3</sub> chirality to the entire tricyclic skeleton in a highly diastereoselective manner. The choice of TMS-acetylene substituted dienophile over against the corresponding diene would ensure appropriate regioselectivity for the IMDA in which the vinyl cyclohexene acts as the diene. The bulky nature of TMS-acetylene also favors *exo*-selectivity in the IMDA. Finally, as described in Scheme 4, the TMS-acetylene moiety serves as a versatile functionality for the eventual incorporation of the heteroaryl systems to provide the target molecules.

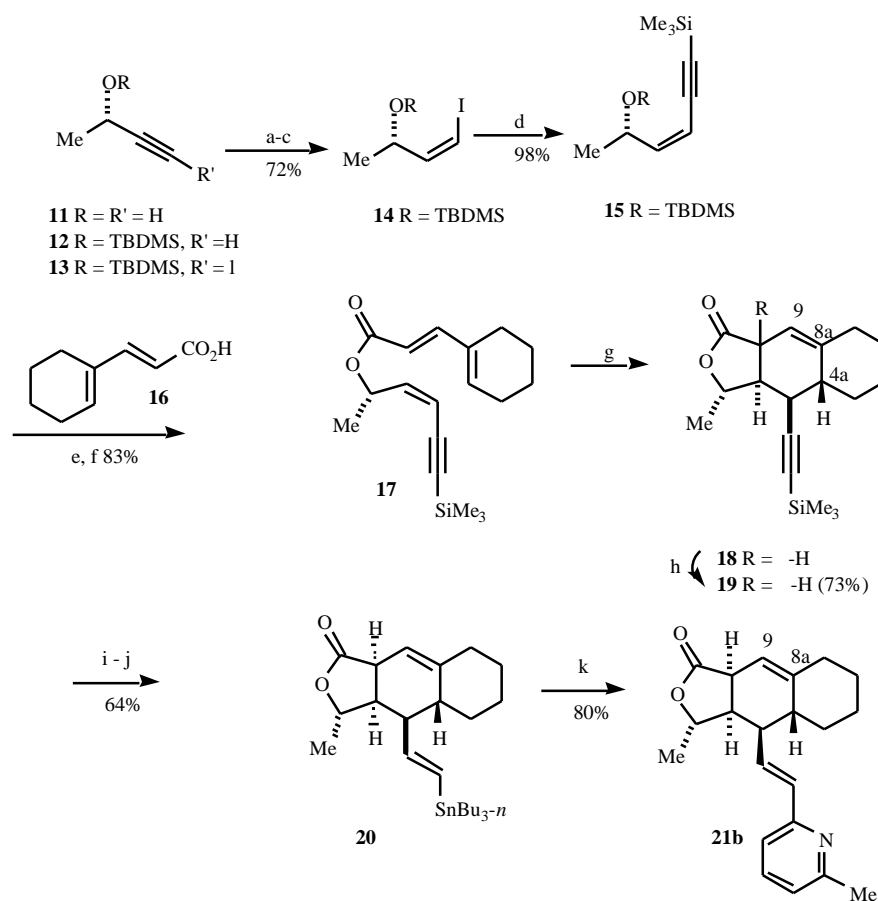


Scheme 3.

The implementation of the above approach to synthesize himbacine-derived PAR-1 antagonists, shown in Scheme 4, commences with commercially available, inexpensive ( $\pm$ )-3-butyn-2-ol (**11**). Protection of the alcohol functionality, followed by treatment with *n*-butyllithium and iodine gave the corresponding iodoacetylene which was reduced to the *cis*-vinyl iodide **14** using (dicyclohexyl)borane. Sonogashira coupling of vinyl iodide **14** with TMS-acetylene gave the ene-yne derivative **15**. Standard deprotection of the TBDMS ether **15**, followed by esterification with the dienoic acid **16**, which was readily prepared from cyclohexanecarboxaldehyde in an overall 41% yield in three steps, gave the Diels-Alder precursor **17**. Thermal cyclization of **17** at 185 °C gave the required *exo*-selective tricyclic acetylene derivative **18** as the predominant product which was readily epimerized to the *cis*-lactone **19** by a brief *in situ* treatment with DBU in an overall 73% yield from the IMDA precursor **17** along with 15% of the endo adduct (not shown). Removal of the TMS group of **19**, followed by hydrostannylation of the corresponding terminal acetylene gave the *trans*-vinyl stannane derivative **20**. Palladium-mediated coupling of appropriate  $\alpha$ -halopyridine derivatives with vinyl stannane **20** gave required targets in excellent yields.

The synthesis outlined in Scheme 4 served as a versatile approach to the SAR development of PAR-1 antagonists. Initially, we explored the SAR of himbacine-derived PAR-1 antagonists in the racemic series due to the ready availability of the starting material. As it later turned out, the presence of the C<sub>8a</sub>-C<sub>9</sub> double bond in **21** had only minimal effect on PAR-1 inhibition. Therefore, it was expedient to explore the SAR using the racemic dehydrohimbacine scaffold followed by eventual optimization in the himbacine series.

The outcome of our initial SAR studies is outlined in Table 3. The monosubstituted pyridine derivative **21a** showed only weak activity against PAR-1. However, additional substitution of the pyridine ring at the 6-position



**Scheme 4.** Reagents and conditions: (a) TBDMSCl, imidazole, DMF; (b) (i) *n*-BuLi, THF; (ii) I<sub>2</sub>; (c) (i) cyclohexene, BH<sub>3</sub>•SMe<sub>2</sub>, pentane; (ii) **13**; (iii) AcOH; (iv) H<sub>2</sub>N(CH<sub>2</sub>)<sub>2</sub>OH; (d) HCCSiMe<sub>3</sub>, PdCl<sub>2</sub>(PhCN)<sub>2</sub>, CuI, piperidine, THF; (e) 2% TFA-MeOH; (f) **16**, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, DMAP, CH<sub>2</sub>Cl<sub>2</sub>; (g) toluene, TEMPO (1% wt equiv), 185 °C, 1.75 h; (h) DBU; (i) K<sub>2</sub>CO<sub>3</sub>, MeOH; (j) *n*-Bu<sub>3</sub>SnH, AIBN, toluene, 120 °C; (k) Pd(PPh<sub>3</sub>)<sub>4</sub>, toluene, 120 °C, 2-bromo-6-methylpyridine.

**Table 3. Structure-Activity Relationship of Dehydrohimbacine-Derived PAR-1 Antagonists**

Entry	R	PAR-1 IC <sub>50</sub> (nM)
<b>21a</b>		3608
<b>21b</b>		350

(Table 3) contd...

Entry	R	PAR-1 IC <sub>50</sub> (nM)
<b>21c</b>		171
<b>21d</b>		351
<b>21e</b>		2252
<b>22</b>		Inactive

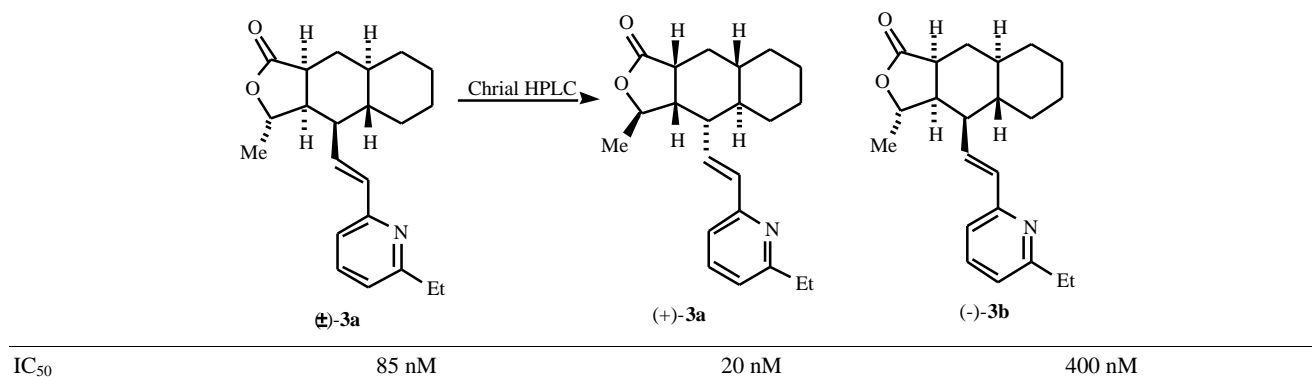


Fig. (4). Enantiospecific binding property of himbacine-based PAR-1 antagonists.

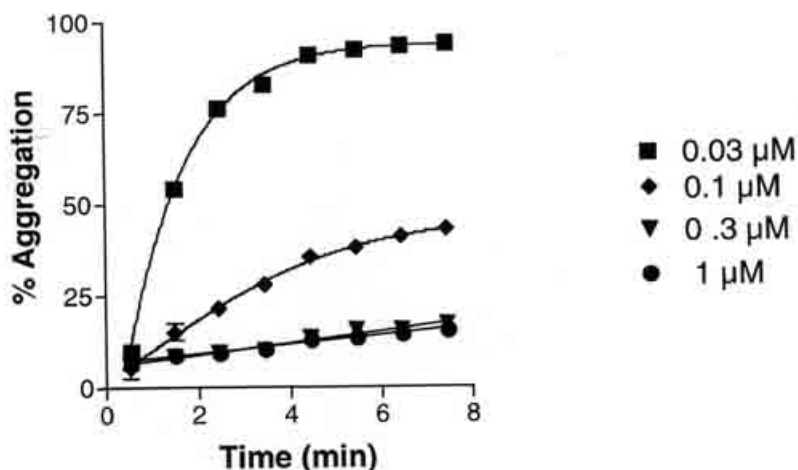


Fig. (5). Inhibition of ha-TRAP induced aggregation of washed human platelets at various concentrations of compound (+)-3a.

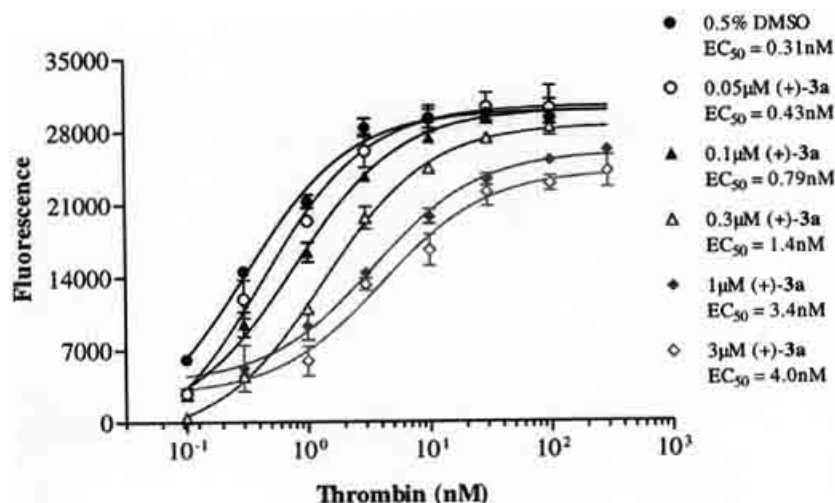
showed dramatic improvement in activity. For example, the 6-methylpyridine derivative **21b** gave an IC<sub>50</sub> of 350 nM. Among the various 6-alkyl derivatives examined, the 6-ethylpyridine derivative **21c** was the most active, giving an IC<sub>50</sub> of 171 nM. Whereas 6-propyl derivative **21d** showed IC<sub>50</sub> comparable to that of 6-methyl derivative **21b**, substitution of this position with bulkier groups resulted in decline of activity as indicated by butyl derivative **21e**. Finally, the *m*-xylyl derivative **22** that is isosteric to **21b** showed no inhibition of the PAR-1 receptor, emphasizing the importance of the substituted vinyl pyridine moiety.

The most active racemic dehydrohimbacine derivative **21c** was further subjected to optimization. Stereoselective reduction of C<sub>8a</sub>-C<sub>9</sub> double bond gave (±)-**3a** which showed an IC<sub>50</sub> of 85 nM in the radioligand binding assay. Chiral chromatographic separation of (±)-**3a** gave two enantiomers (+)-**3a** and (-)-**3b**, which showed distinctly different affinity for the thrombin receptor (Fig. 4). The (+)-isomer **3a** gave an IC<sub>50</sub> of 20 nM whereas the (-)-isomer **3b** gave an IC<sub>50</sub> of 400 nM. Assignment of the absolute stereochemistry was carried out by chiral synthesis of the (+)-enantiomer from (*R*)-3-butyn-2-ol and the (-)-enantiomer from (*S*)-3-butyn-2-ol according to Scheme 4. This study indicates that the *ent*-

himbacine absolute stereochemistry is preferred for PAR-1 antagonism.

Compound (+)-**3a** was subjected to further studies. In the radioligand binding assay, this compound showed a K<sub>i</sub> value of 12 nM against the PAR-1 receptor. In the ha-TRAP induced human platelet aggregation, this compound showed potent dose-dependent inhibition with an apparent IC<sub>50</sub> of 70 nM (Fig. 5), making it one of the most potent thrombin receptor antagonists reported. Similarly, in the cytosolic intracellular Ca<sup>+2</sup> mobilization assay, this compound showed potent, dose-dependent inhibition of Ca<sup>+2</sup> mobilization (Fig. 6).

In summary, we have identified a number of non-peptide thrombin receptor antagonists in three different structural series. We have demonstrated that these compounds inhibit [<sup>3</sup>H]ha-TRAP in a competitive manner. They also inhibit ha-TRAP and thrombin induced platelet aggregation in a dose dependent manner. The functional properties of selected members of these compounds were further studied in the cytosolic Ca<sup>+2</sup> transient measurement in hCASM and thrombin-stimulated thymidine incorporation in hCASM. In these assays, these compounds showed potent dose-dependent inhibition of activity.



**Fig. (6).** Inhibitory effects of (+)-3a on concentration-dependent stimulation of calcium transients induced by thrombin in hCASMIC. Varying concentrations of (+)-3a were added 30 min before the addition of increasing concentrations of thrombin. Key: (●) vehicle (0.2% DMSO); (○) 0.05 μM (+)-3a; (▲) 0.1 μM (+)-3a; (◻) 0.3 μM (+)-3a; (◆) 1 μM (+)-3a; and (◇) 3 μM (+)-3a.

## CONCLUSION

The prototypical PAR receptor, known as PAR-1 or the thrombin receptor, is a highly promising therapeutic target for treating a variety of cardiovascular disorders such as unstable angina, acute myocardial infarction, stroke, and restenosis. Since thrombin is the most potent activator of platelets, a thrombin receptor antagonist should have a strong antiplatelet effect under conditions in which thrombin stimulated platelet activation is critical. In addition, the pharmacological profile of the thrombin receptor antagonist presents a unique opportunity for the treatment of restenosis since the underlying etiology of restenosis is characterized by inflammatory and proliferative processes stimulated by thrombin's cellular activation. Furthermore, since a thrombin receptor antagonist is specific for the cellular actions of thrombin, and does not affect fibrin generation, it should have a better safety profile than GpIIb/IIIa (fibrinogen receptor) antagonists with regard to hemorrhagic side effects.

The practicality of effectively blocking thrombin receptor activation by the tethered ligand has been debated since an antagonist has to compete in a bimolecular sense with a resident internal ligand that enjoys considerable entropic advantage.<sup>15</sup> Although the final answer to this question will come only in efficacy models in the in vivo settings, the following information suggests that this is an achievable goal. High affinity peptide and non-peptide PAR-1 antagonists have been reported. Several of these inhibit thrombin and peptide agonist-induced platelet aggregation. The potential antirestenosis utility for a thrombin receptor antagonist has been established in a proof-of-principle rat restenosis model [16], and studies performed in baboons with a thrombin receptor antibody indicates that arterial thrombosis can be inhibited by inactivation of PAR-1 without affecting template bleeding time [17]. While these studies suggest the therapeutic potential of a thrombin receptor antagonist, the data presented here indicate that

substantial progress has been made toward the identification of potent, low molecular weight thrombin receptor antagonists. For example, the himbacine-derived compound (+)-3a is the most potent non-peptide thrombin receptor antagonist reported to date in radioligand binding assay as well as functional assays.

## ACKNOWLEDGEMENT

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