Research Article

Synthesis of Novel Peptide Inhibitors of Thrombin-induced Platelet Activation

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Inhibitors of the activation of platelet aggregation have promise as important therapeutic agents for the management of acute coronary syndrome (ACS). Platelet activation by thrombin, a serine protease, occurs by binding to and cleavage of the extracellular N-terminal domains of protease-activated receptors 1 and 4 (PAR1 and PAR4). The proteolysis of the PARs exposes new tethered ligands that then signal through transmembrane domains to initiate platelet activation as a downstream effect. A pentapeptide cleavage product of bradykinin with the sequence Arg-Pro-Pro-Gly-Phe serves as a thrombin inhibitor by blocking α- and γ-thrombin-induced platelet aggregation. Analogs of RPPGF have been prepared that result in improved inhibition of thrombin activation of platelets. Specific amino acid residues required for activity against platelet aggregation have been identified, and a lead compound, rOicPaPhe(p-Me)-NH$_2$ (FM19), has been developed. FM19, which completely inhibits threshold γ-thrombin-induced platelet aggregation at a concentration of 16 ± 4 μM, represents an important lead compound in the development of inhibitors of thrombin-mediated platelet aggregation for treatment of ACS.

Key words: bradykinin, protease-activated receptor, platelet aggregation, thrombin, thrombin receptor

Acute coronary syndrome (ACS) refers to a number of conditions, from unstable angina to myocardial infarction (MI), which most often develop as a result of reduced coronary artery blood supply because of ruptured atherosclerotic plaque (1). Approximately 90% of MIs result from an acute thrombus that obstructs an atherosclerotic coronary artery (1). Therefore, modulating the activity of the hemostatic system is both treatment and prevention of these serious and too often fatal medical conditions.

Thrombin has an important role in hemostasis and thrombosis by being the main clotting enzyme as well as a potent platelet activator (2). Thrombin has two substrates on platelets, protease-activated receptors 1 and 4 (PAR1 and PAR4), that when stimulated lead to platelet activation. The PARs are G-protein-coupled receptors that have a novel mechanism in which an extracellular proteolytic cleavage event is translated into a transmembrane signal. These receptors carry their own ligand, which remains cryptic until an N-terminal fragment of the receptor is cleaved by thrombin (3). Once activated by thrombin-mediated proteolysis, these G-protein-coupled receptors' tethered ligands bind intramolecularly to induce signaling to activate platelets (4). To date, only inhibitors to arachidonic acid- or ADP-induced platelet aggregation have been developed for clinical use. Development of inhibitors to thrombin-induced platelet activation would advance the field.

Bradykinin, a nonapeptide, is released from the plasma proteins, high- and low-molecular weight kininogen through the action of plasma kallikrein (5). Bradykinin increases vascular permeability, dilates blood vessels, contracts non-vascular smooth muscle, and causes pain. This short-lived vasodilator is degraded by angiotensin-converting enzyme to produce a pentapeptide with sequence Arg-Pro-Pro-Gly-Phe (RPPGF) (6). This pentapeptide, along with kininogen and bradykinin, inhibits thrombin-induced platelet activation and aggregation by interacting with the active site of thrombin and with the thrombin cleavage sites of PAR1 and PAR4 receptors (7,8). Therefore, RPPGF and its analog rOicPGF-NH$_2$ are leads for the development of a selective and potent inhibitor of thrombin-induced platelet activation (9,10). This study reports substitutions made to the rOicPGF-NH$_2$ sequence designed to produce a more potent thrombin receptor activation inhibitor for the management of ACS, and describes the discovery of a novel lead compound.

Materials and Methods

Peptides and reagents

Standard single-letter abbreviations are used to describe L-amino acids and lowercase letters are used to represent D-amino acids. RPPGF and rOicPGF were synthesized by Multiple Peptides Systems, Inc. (San Diego, CA, USA). The remaining peptides were prepared by standard solid-phase peptide synthesis methods on a Applied Biosystems, Inc. (Foster City, CA, USA) Model 431A peptide synthesizer (11,12). The peptides were assembled on peptide amide linker polyethylene glycol polystyrene (PAL-PEG-PS) resin (Applied Biosystems, Warrington, UK) using standard Fmoc-based chemistry (13).

The protected amino acids Fmoc-D-Arg (Pbf), Fmoc-Phe(p-Me), Fmoc-Phe(p-Br), Fmoc-Phe(p-I), Fmoc-Phe(p-NO$_2$), Fmoc-Pro, Fmoc-Oic, Fmoc-D-Ser(t-Bu), Fmoc-Lys(biotin), and Fmoc-D-Ala were obtained from Advanced ChemTech (Louisville, KY, USA). Unless otherwise stated, all reagents were HPLC grade and purchased from Fisher Scientific (Fair Lawn, NJ, USA).

The synthesis occurred in a cycle that was initiated by the swelling of the resin (0.25 mmol) in dichloromethane (DCM) followed by de-protection or removal of the Fmoc group from the resin with piperidine (Aldrich, Milwaukee, WI, USA), followed by extensive solvent wash and, subsequently, by coupling of Fmoc-protected amino acid (1 mmol). Coupling was facilitated by o-benzotriazol-1-yl-N,N,N',N'-tetramethyl uronium hexafluorophosphate (HBTU; Applied Biosystems), which was dissolved in a solution of 1-hydroxybenzotriazole (HOBt; Applied Biosystems) and N,N-dimethylformamide (DMF). The protected amino acid was dissolved in the HBTU/HOBt solution along with additional N-methylpyrrolidone (NMP). The reaction was carried out in the presence of 2.0 M N,N-diisopropylethylamine (DIEA; Applied Biosystems), which created the basic environment necessary for the activation of the amino acid residue (14). The activated amino acid reacted with the deprotected amino-terminal of the growing peptide chain. The final step in each coupling cycle involved acetylation of unreacted amino groups by addition of 10-fold excess of 0.5 M acetic anhydride, 0.125 M DIEA, and 0.015 M HOBt in NMP. This sequence of steps was repeated until the full-length, resin-bound peptide was completed. The resin was filtered and washed with DCM and ethyl alcohol (EtOH) to complete the solid-phase synthesis.

Trifluoroacetic acid/H$_2$O/thioanisole/ethylenedithiol solution (9:0.5:0.25:0.25, v/v/v/v; Pierce, Rockford, IL, USA)/(Applied Biosystems) was used to cleave the peptide from the resin and simultaneously remove the side chain-protecting groups. The peptide solution was filtered from the resin and then subjected to preparative reverse-phase high-performance liquid chromatography (RP-HPLC) to afford the target peptide. The HPLC conditions were: column, Vydac C18, 2.5 × 22 cm; mobile phase, (A) water with 0.1% TFA, (B) aceto-nitrile with 0.1% TFA; gradient, (A/B) from 100/0 to 60/40 over 80 min; flow rate, 10 mL/min; detection, UV at 230 nm. The purity of all peptides was ≥99% as analyzed by HPLC. Final product molecular weight was confirmed by electrospray mass spectrometry (ThermoFinnigan, San Jose, CA, USA).

Platelet aggregation

Each peptide was examined for its ability to inhibit γ-thrombin-induced platelet aggregation of human platelet-rich plasma (PRP) as described by Hasan et al. (8,10). Fresh whole blood from normal human donors was collected into a syringe containing 3.8% sodium citrate, to a final concentration of 0.38% sodium citrate (1:9 cit-rate/blood). Whole blood was centrifuged at 180 × g (Beckman GRP Centrifuge, Schaumburg, IL, USA) for 10 min at room temperature to prepare PRP. Platelet-poor plasma (PPP) was prepared by centrifugation of PRP at 1000 × g for 20 min at room temperature or by centrifugation at 10 000 × g for 2 min (Microcentrifuge E, Beckman Instruments, Palo Alto, CA, USA). The platelets were counted on a Coulter counter (Model Z, Coulter Electronics, Hialeah, FL,

USA), and PRP was adjusted to a platelet count of 2.5×10^8 plate-lets/mL, using PPP prior to all platelet aggregation studies. A Chro-nolog dual-channel aggregometer (Havertown, PA, USA) was used to assess platelet aggregation by recording the increase in light transmittance through a stirred suspension of PRP maintained at 37 °C in the cuvette. Variable concentrations of γ-thrombin (Haema-tologic Technologies, Essex Junction, VT, USA) were added to the PRP to determine the threshold concentration for maximal percent-age aggregation, i.e. the minimal concentration of the agonist to induce full aggregation and secretion. γ-Thrombin (Haematologic Technologies; approximately 3000 U/mg specific activity) was used for these studies because this form of the enzyme is unable to bind and proteolyze plasma fibrinogen in plasma to produce a clot. Pep-tides were dissolved in fresh HEPES-Tyrodes buffer (pH = 7.40) and added at concentrations ranging from 5 μM to 1 mM to the PRP in the cuvette, allowing the baseline to stabilize. Platelet aggregation was initiated by the addition of threshold amounts of γ-thrombin (usually 30–70 nM for human platelets) determined for that donor on the day of examination. The aggregation was allowed to proceed for 5 min and the minimal concentration of each peptide to achieve 100% inhibition of platelet aggregation (IC$_{100}$) was determined.

Results and Discussion

To develop analogs related to RPPGF with increased potency for inhibition of thrombin-induced platelet aggregation, we focused our initial efforts on substituting residues at positions 4 and 5. Previous work employing both combinatorial methods and rational drug design indicated that the substitution of the first residue with D-Arg and replacement of Pro^2 by Oic, known to improve potency of bra-dykinin antagonists; while maintaining proline in the third position of the sequence, led to pronounced improvements in potency, and resulted in the lead compound, rOicPGF-NH$_2$ (TH146) (10,15,16). The goal of the current efforts was to optimize the remaining positions by preparing analogs with substitutions at Xxx^4 and Xxx^5. The results of these substitutions are summarized in Tables 1 and 2, where data are reported as the minimal concentration of the inhib-itor to completely block threshold γ-thrombin-induced platelet aggregation (7,8,10). Several previously synthesized and reported compounds are also included for comparison.

Table 1 presents compounds with replacements for Gly at position 4 of rOicPGF-NH$_2$ (TH146). As glycine has no side chain and can assume conformations allowed for both L- and D-amino acids, alan-ine, the most conservative substitution for glycine, was examined in both L- and D-configurations (compounds **1** and **2**, respectively). As seen in Table 1, compound **2**, which showed 100% inhibition of platelet aggregation at 56 μM, is 10-fold more potent than com-pound **1** and 2.5-fold more potent then TH146 in γ-thrombin-induced platelet aggregation.

The successful results observed during the testing of **2**, encouraged us to explore further D-amino acid substitutions in position 4. Among the remaining D-amino acid-substituted analogs, compound **3**, rOic-PnF-NH$_2$ showed a modest improvement over TH146 with 100% inhi-bition of platelet aggregation at 83 μM, slightly less potent than **2**. The remaining analogs in this series had similar activity independent

Table 1: Modifications at position 4 with phe at position 5

Compounds	Sequence	Inhibition of threshold platelet aggregation (μM ± SEM[a])
RPPGF[b]	RPPGF	680 ± 280
TH146	rOicPGF-NH$_2$	140 ± 20
1	rOicPAF-NH$_2$	550 ± 166
2	rOicPaF-NH$_2$	56 ± 16
3	rOicPnF-NH$_2$	83 ± 17
4	rOicPkF-NH$_2$	133 ± 33
5	rOicPfF-NH$_2$	133 ± 33
6	rOicPpF-NH$_2$	167 ± 33
7	rOicPlF-NH$_2$	300 ± 100
9	rOicPdF-NH$_2$	567 ± 233
10	rOicPqF-NH$_2$	400 ± 100
11	rOicP-(D-Abu)-F-NH$_2$	275 ± 75
12	rOicP-(D-Orn)-F-NH$_2$	250 ± 87
13	rOicP-(D-Nle)-F-NH$_2$	275 ± 75

[a]Data presented are the mean ± SEM of $n > 3$ independent experiments. The values given represent the minimal concentration of peptide necessary to inhibit 100% of threshold γ-thrombin-induced platelet aggregation (IC$_{100}$).
[b]From Hasan *et al.* (7).

Table 2: Modifications at position 5 with gly at position 4

Compounds	Sequence	Inhibition of threshold platelet aggregation (μM ± SEM[a])
RPPGF[b]	RPPGF	680 ± 280
TH146	rOicPGF-NH$_2$	140 ± 20
14	rOicPG-2-Nal-NH$_2$	50 ± 0
15	rOicPGE-NH$_2$	NE
16	rOicPG-Nle-NH$_2$	NE
17	rOicPGF(p-F)-NH$_2$	200 ± 0
18	rOicPGf-NH$_2$	833 ± 144
19	rOicPGW-NH$_2$	350 ± 87
20	rOicPGH-NH$_2$	NE
21	rOicPG-1-Nal-NH$_2$	200
22	rOicPGR-NH$_2$	NE
23	rOicPGY-NH$_2$	300 ± 100

[a]Data presented are the mean ± SEM of $n > 3$ independent experiments. The values given represent the minimal concentration of peptide necessary to inhibit 100% of threshold γ-thrombin-induced platelet aggregation (IC$_{100}$). NE represents no effect or ≥1000 μM activity.
[b]From Hasan *et al.* (7).

of the chemical nature of the residue side chain. Consequently, the trend observed in these peptides was that the requirement for residue 4 seemed to be primarily related to the configuration of the amino acid residue, with the actual side chain having a relatively modest effect. A possible explanation is that the D-stereochemistry moves the side chain 'out of the way' while helping to orient the rest of the molecule. This assumption would be consistent with the optimal activity displayed by **2**, with the small alanine side chain.

In parallel with the examination of varying Xxx^4, we also varied Xxx^5 of the lead compound TH146. The data for the rOicPG-Xxx^5-NH$_2$ series are presented in Table 2, in which it is clearly observed that L-amino acids are preferred over D-amino acids. This trend is exemplified in the sharp decrease in activity in compound **18**, with

D-phenylalanine at position 5 (IC$_{100}$ = 833 μM), in comparison with TH146 (IC$_{100}$ = 140 μM). In addition, it appears that larger aromatic groups are preferred while aliphatic and polar groups are not well tolerated. The most potent compound in this series, compound **14**, replaces Phe^5 with 2-Nal^5 in rOicPGXxx^5-NH$_2$ resulting in a three-fold improvement over TH146 (IC$_{100}$ = 50 μM).

Following the results from our two initial series, we attempted to combine the best substitutions observed in the singly modified Xxx^4 and Xxx^5 series, but unfortunately the results do not appear to be additive. On the contrary, as shown in Table 3, the projected best analog, compound **25** is only slightly more potent than TH146 (IC$_{100}$ = 100 μM). The explanation for the observed result may be related to steric and conformational restrictions imposed on the doubly substituted peptide, with a less flexible backbone (due to D-alanine at position 4) and the bulky side chain of 2-Nal. Thus, we chose to examine other possible aromatic replacements for 2-Nal at position 5.

Consequently, as shown in Table 3, we introduced several variations of Phe that, like the successful 2-Nal^5 substitution, were larger, aromatic, and lipophilic. In the initial analogs of the rOicPG-Xxx^5-NH$_2$ series, we chose to use Phe derivatives as a means of probing the requirements for interaction with the target receptor. Phe is present in the original lead, RPPGF, and, as seen in Table 2, the aromatic nature of Phe^5, appears to be essential for inhibition of platelet activation. As a first group, we chose to use small (methyl), intermediate (chloro), and large (biphenyl) lipophilic substituents at the *para*-position of the phenyl ring of phenylalanine. In this initial small sample of synthesized analogs, there was a clear preference for the smallest substituent at the *para*-position of Phe. The *p*-methylphenylalanine containing compound **27** was 3.5-fold more potent than compound **2**, 42.5-fold more potent than RPPGF, and had a ninefold increase in potency relative to TH146 in γ-thrombin-induced platelet aggregation.

Table 4 presents the results from our attempts to examine the key features of **27** that are required for its anticoagulant activity. In this series, we selected a variety of Phe derivatives with a wide range of substituents. We also examined several analogs that retained the *p*-methylphenylalanine at the fifth position, but included modifications at the fourth position that had shown activity in the rOicP-Xxx^4-Phe(*p*-Me)-NH$_2$ series. As seen in Table 4, several

Table 3: Modifications at position 5 with D-ala in position 4

Compounds	Sequence	Inhibition of threshold platelet aggregation (μM ± SEM[a])
RPPGF[b]	RPPGF-NH$_2$	680 ± 280
TH146	rOicPGF-NH$_2$	140 ± 20
24	rOicPaF-NH$_2$	56 ± 16
25	rOicPa-2-Nal-NH$_2$	100 ± 35
26	rOica-Bip-NH$_2$	200 ± 100
27 (FM19)	rOicPaF(*p*-Me)-NH$_2$	16 ± 4

[a]Data presented are the mean ± SEM of $n > 3$ independent experiments. The values given represent the minimal concentration of peptide necessary to inhibit 100% of threshold γ-thrombin-induced platelet aggregation (IC$_{100}$).
[b]From Hasan *et al.* (7).

Table 4: Modifications to positions 4 and 5 compared with **27**

Compounds	Sequence	Fold decrease in the inhibition of platelet aggregation potency versus **FM19**
27 (FM19)	rOicPaF(*p*-Me)-NH$_2$	1[a]
28	rOicPaF(*p*-Cl)-NH$_2$	2.75
29	rOicPsF(*p*-Me)-NH$_2$	0.81
30	rOicPnF(*p*-Me)-NH$_2$	4
31	rOicPa-Hfe-NH$_2$	8
32	rOicPaF(*p*-NH$_2$)-NH$_2$	8
33	rOicPaF(*p*-Br)-NH$_2$	1.1
34	rOicPa(*p*-But)-NH$_2$	2
35	rOicPaF(*p*-CN)-NH$_2$	2
36	rOicPaF(*p*-I)-NH$_2$	1
37	rOicPaF(2-Me)-NH$_2$	2
38	rOicPaF(3-Me)-NH$_2$	4
39	rOicPaF(*p*-NO$_2$)-NH$_2$	3
40	rOicPa-Phg-NH$_2$	2

[a]The data represent the fold increase in concentration of the peptide necessary to inhibit threshold γ-thrombin-induced platelet aggregation when compared with compound **27** (**FM19**) (IC$_{100}$ = 16 ± 4 μM).

analogs display potency comparable with that of **27**. In particular **29**, **33**, and **36** are essentially equipotent with **27**. Although incomplete, these initial structure–activity relationship (SAR) data suggest that lipophilic *para*-substituents on Phe^5 lead to favorable results and that, as observed in Table 1, small D-amino acids are preferred in position 4 of the peptide. Further SAR studies are in progress.

Conclusions

Several trends were observed in this series of peptides synthesized with the aim of inhibiting thrombin-mediated platelet aggregation. The fourth position of the peptide rOicPGF-NH$_2$ has a preference for D-amino acids, but is relatively indiscriminant with regard to the actual amino acid residue and its side chain properties. The presence of an aromatic ring on the side chain of the amino acid in position 5 is preferred over all other amino acid side chains, with Phe(*p*-Me) displaying the best activity thus far. In compound **27**, [rOicPaPhe(*p*-Me)-NH$_2$], known as **FM19**, we have developed a more potent inhibitor (IC$_{100}$ = 16 μM) of γ-thrombin-induced platelet aggregation, which represents an order of magnitude improvement over previous RPPGF-related inhibitors. Further studies are underway to extend the SARs of **FM19**-related analogs and elucidating its mechanism of action.

Acknowledgments

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