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Structure-Based Design of Residue 1 Analogs of the Direct Thrombin Inhibitor Pentapeptide FM 19

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Myocardial ischemia and other acute coronary syndromes are leading causes of death worldwide, and often result from a thrombus that blocks an atherosclerotic coronary artery. A key enzyme in thrombus formation is the serine protease thrombin, which is responsible for both the conversion of soluble fibrinogen into insoluble fibrin, as well as the activation of the GPCRs, PAR1 and PAR4, which stimulate platelet aggregation. Thus, thrombin is an attractive target for anticoagulant and antithrombotic therapy. Previous studies in our laboratory led to the development of lead compound FM 19 (D-Arg-Oic-Pro-D-Ala-Phe(*p*-Me)-NH_2), which shows modest potency as a thrombin inhibitor. The recently determined X-ray structure of FM 19 in the active site of thrombin has revealed potential sites for modification to improve potency. This study reports replacements to the first residue (D-Arg^1) of FM 19, which seek to improve potency by removing the N-terminal amine to eliminate an adverse electrostatic interaction, and alterations to the length of the side chain to eliminate an unfavorable eclipsed conformation observed in the X-ray structure. This study produced two compounds, 1 and 9, with improved α-thrombin inhibition (IC_{50} values of 0.66 ± 0.20 μM and 0.57 ± 0.12 μM, respectively).

Key words: antithrombotic therapy, peptide synthesis, protease-activated receptor, structure-based design, thrombin

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Acute coronary syndrome (ACS) is a broad term that can refer to many different disease states, such as unstable angina and myocardial infarction (MI), which is a leading cause of death worldwide (1,2). MIs occur when blood supply to the heart is interrupted, which is most commonly caused by the formation of a thrombus that blocks a coronary artery (1,3,4). A key enzyme in thrombus formation is the serine protease thrombin, which is responsible for two major clotting events. First, thrombin cleaves an extracellular portion of the protease-activated receptors 1 and 4 (PAR1 and PAR4), which are G protein-coupled receptors (GPCRs) found on platelets. Once this fragment is cleaved, the newly uncovered N-terminus binds intramolecularly to stimulate platelet activation (3). Secondly, thrombin converts soluble fibrinogen into insoluble fibrin, the protein that cross-links with platelets to form a thrombus (1,5). Thus, thrombin is an attractive target for both antiplatelet and antithrombotic therapy.

Bradykinin, a nonapeptide cleavage product of high-molecular-weight kininogen with the sequence RPPGFSPFR (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg), increases vascular permeability and has cardioprotective effects (6–8). Bradykinin can be further cleaved by angiotensin-converting enzyme to produce a pentapeptide with the sequence RPPGF (Arg-Pro-Pro-Gly-Phe) (4). RPPGF has been shown to inhibit the actions of thrombin in two ways (4). First, RPPGF binds to the active site of thrombin in a retro-binding fashion. That is, RPPGF binds to the thrombin active site in an opposite sense (in terms of linear sequence) compared to thrombin substrates (4). Secondly, RPPGF has been shown to bind to the thrombin cleavage site on PAR1, preventing cleavage by thrombin, and therefore inhibiting platelet activation (4). Thus, peptide RPPGF serves as a starting point for the development of a direct thrombin inhibitor, as well as an inhibitor of platelet activation.

SAR studies were carried out to follow-up on RPPGF antithrombotic activity and culminated in the development of lead compound, FM 19, with the sequence D-Arg-Oic-Pro-D-Ala-Phe(*p*-Me)-NH_2, where Oic is ((2*S*,3*aS*,7*aS*)-octahydroindole-2-carboxylic acid) (Figure 1) (9). Although FM 19 is more potent than RPPGF (K_i = 4.4 μM versus 1.75 mM), FM 19 is still only a modest inhibitor of thrombin activity (10). The crystal structure of FM 19 in thrombin's active site has recently been determined, showing that, like RPPGF, FM 19 is a retrobinder (Figure 2) (10). This crystal structure provides insight into potential modifications that can be made to the FM 19 structure to increase binding interactions and potency. The crystal structure shows that the side chain of the D-Arg^1 residue adopts a high-energy eclipsed conformation, which places the positively charged guanidino group of the side chain in close proximity to the N-terminal amine, creating an unfavorable electrostatic interaction. We report here initial replacements to the

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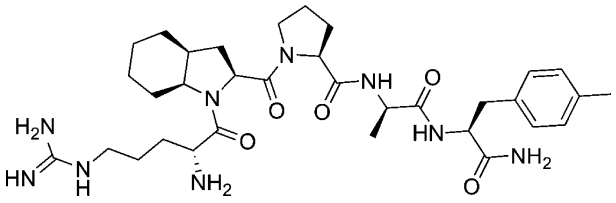

Figure 1: Structure of FM 19 (D-Arg-Oic-Pro-D-Ala-Phe(*p*-Me)-NH_2

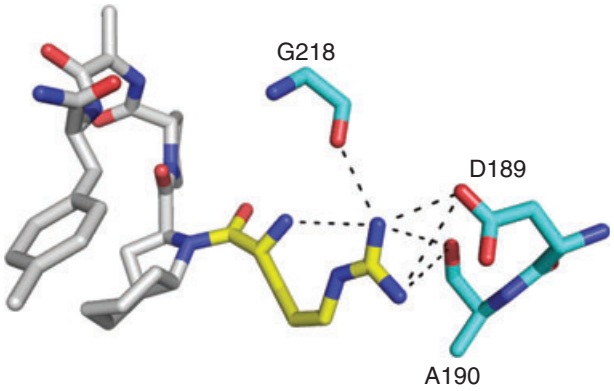

Figure 2: Crystal structure of FM 19 in thrombin's active site. The D-Arg^1 residue (yellow) interacts with G218, D189 and A190 of thrombin (cyan). PDB: 3BV9, 1.80 Å resolution.

D-Arg^1 residue of the FM 19 sequence (Figure 3) aimed at eliminating the unfavorable electrostatic interaction, and explore altering the side-chain length in an attempt to alleviate the eclipsed conformation. Conversely, we also report an initial application of conformational restriction to this side chain, to stabilize the eclipsed conformation.

Materials and Methods

Reagents

NMP (*N*-methyl-2-pyrrolidinone) and diisopropylethylamine were purchased from Advanced Chem Tech (Louisville, KY, USA). Piperidine, trifluoroacetic acid for cleavage and deprotection, dimethylformamide, triethylamine and dioxane were purchased from Sigma-Aldrich (St Louis, MO, USA). Diethyl ether, ethyl acetate and HPLC grade solvents (water, acetonitrile and trifluoroacetic acid) were purchased from Fisher Scientific (Pittsburg, PA, USA). Dichloromethane was purchased from Acros Organics (Geel, Belgium).

Amino acids and peptides

All Fmoc-protected amino acids (Fmoc-Phe(4-Me)-OH, Fmoc-Oic-OH, Fmoc-Pro-OH, Fmoc-D-Ala-OH·H_2O and Fmoc-Gly-OH) were purchased from Advanced Chem Tech (Louisville, KY, USA). Boc-7-aminoheptanoic acid (*N-tert*-butoxycarbonyl-7-aminoheptanoic acid) was purchased from Anaspec (San Jose, CA, USA). 4-Aminobutyric acid and 6-aminohexanoic acid were purchased from Eastman Kodak Co. (Rochester, NY, USA). 5-Aminovaleric acid and β-alanine were purchased from Sigma-Aldrich. The *p*-aminobenzoic acid was a generous gift from the laboratory of Dr. Scott Larsen, University of Michigan, College of Pharmacy.

Amine protection

The free amino groups on the side chains of 5-aminovaleric acid, β-alanine, 4-aminobutyric acid, 6-aminohexanoic acid and *p*-aminobenzoic acid were protected before peptide synthesis with a Boc (*N-tert*-butoxycarbonyl) group as previously reported (11).

Synthesis of N, N'*-di-Boc-4-guanidinobenzoic acid*

N, *N*'-di-Boc-4-guanidinobenzoic acid was synthesized using a slight modification of a previously described procedure (12). One equivalent of *N*, *N*'-di-Boc- *N*'-triflylguanidine (Fluka, Buchs, Switzerland) was dissolved in dioxane in the presence of a threefold excess of triethylamine, and a 10% excess of *p*-aminobenzoic acid was added. After 3 h, an additional twofold excess of triethylamine was added, and the reaction was stirred for 8–19 days at room temperature. The solution was then concentrated *in vacuo* and dissolved in ethyl acetate and H_2O. The aqueous layer was removed, acidified to pH 1.5, and extracted 5 times with ethyl acetate. Saturated $NaHCO_3$ was added to the combined organic layers, prompting precipitation of *N*, *N*'-di-Boc-4-guanidinobenzoic acid as a white solid. This product was then used to make compound **9**.

Peptide synthesis

Peptides were synthesized using standard solid phase Fmoc (fluorenylmethyloxycarbonyl) chemistry on a CS Bio CS336X Peptide Synthesizer (CS Bio Company, Menlo Park, CA, USA), using previously described protocols (13). Rink resin (Advanced Chem Tech, Louisville, KY, USA) was used to produce all peptides as C-terminal amides. A 20% solution of piperidine in NMP was used to remove the Fmoc-protecting group from the Rink resin linker, and again to remove the Fmoc-protecting group after each coupling cycle. Coupling was performed using a fourfold excess of amino acid and a solution of 0.4 M hydroxybenzotriazole (HOBt; Advanced Chem Tech, Louisville,

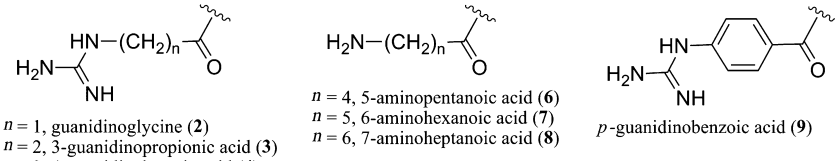

Figure 3: Structures of replacements to the D-Arg^1 residue

KY, USA) and O-benzotriazole- *N*, *N*, *N*', *N*'-tetramethyl-uronium-hexafluoro-phosphate (HBTU; Advanced Chem Tech, Louisville, KY, USA) in dimethylformamide, in the presence of diisopropylethylamine. After the synthesis was complete, the resin was washed with NMP and then dichloromethane, and dried. The peptides were cleaved from the resin and side-chain-protecting groups removed after treatment for 3–4 h with a cleavage cocktail consisting of 9.5 mL trifluoroacetic acid and 0.5 mL H_2O. The solution was concentrated *in vacuo*, and peptides were precipitated using cold diethylether. The filtered crude material was then purified using a Waters semi-preparative HPLC (Waters Corporation, Milford, MA, USA) with a Vydac Protein and Peptide C_{18} column, using a linear gradient of 0% or 10% Solvent B (0.1% trifluoroacetic acid in acetonitrile) in Solvent A (0.1% trifluoroacetic acid in water) to 50% Solvent B in Solvent A, at a rate of 1% per minute. The identity of each peptide was determined by mass spectrometry using a Finnigan LCQ mass spectrometer (ThermoFinnigan, San Jose, CA, USA) in positive mode. The purity of all final peptides was determined using a Waters Alliance 2690 Analytical HPLC (Waters Corporation, Milford, MA, USA) and Vydac Protein and Peptide C_{18} reverse phase column, using a linear gradient of 0–70% Solvent B in Solvent A in 70 min.

Guanylated compounds 1–5

Precursors of **1**–**5** were synthesized first as peptides containing an amino group in the place of the desired guanidino group. Each peptide was then cleaved from the resin and had the Boc-protecting group removed, as described earlier. Guanylation of the primary amine was then carried out similarly as previously described (12). One equivalent of *N*, *N*'-di-Boc-*N*'-triflylguanidine was dissolved in dioxane in the presence of a threefold excess of triethylamine, and a 10% excess of the amine-containing precursor peptide was added. After 3 h, an additional twofold excess of triethylamine was added, and the reaction was stirred for another 7–21 h at room temperature. The solution was then concentrated *in vacuo*, dissolved in ethyl acetate and washed twice each with 2 M $KHSO_4$ and saturated $NaHCO_3$. After drying over anhydrous $MgSO_4$, the ethyl acetate was removed *in vacuo*, and the remaining residue was treated at room temperature with a deprotection solution consisting of 9.5 mL trifluoroacetic acid and 0.5 mL H_2O for 3–4 h. This solution was then concentrated *in vacuo*, and the peptide was precipitated with diethylether and filtered. The crude material was then purified, identified, and final purity was determined as described earlier.

Compound 9

Compound **9** followed the general peptide synthesis protocol described earlier for the first four coupling and Fmoc-deprotection cycles. A twofold excess of *N,N*'-di-Boc-4-guanidinobenzoic acid was dissolved in NMP, and to this solution was added a fourfold excess of 2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU, Advanced Chem Tech, Louisville, KY) and a 26-fold excess of diisopropylethylamine. The solution was then added to the tetrapeptide resin and allowed to shake overnight. The resin was then rinsed with dichloromethane and dried, and the peptide cleaved, precipitated and purified as described earlier.

Thrombin inhibition

Assays to measure the ability of synthetic peptides to inhibit human α-thrombin's (Haematologic Technologies, Essex Junction, VT, USA) cleavage of the chromogenic substrate Sar-Pro-Arg-pNA, where pNA is *p*-nitroanilide (Bachem, Torrance, CA, USA) were adapted from a previously described procedure (14). Experiments were carried out in triplicate in 96-well plates, in the presence or absence of synthetic peptide inhibitors. Controls included no enzyme, no inhibitor, and lead compound FM 19. Enzyme was preincubated with varying concentrations of inhibitor in assay buffer (10 mM Tris, 150 mM NaCl, pH 7.6) for 5 min at 37 °C, and the experiment was started with the addition of substrate. After 10 min, the OD_{405} was plotted as a function of the negative log of the inhibitor concentration to generate a dose–response curve using GraphPad Prism[a]. From these data, an IC_{50} value was determined. Each compound was tested in at least three independent experiments, and the average and standard deviation are reported.

Results and Discussion

Nine compounds with replacements for the D-Arg[1] residue in the FM 19 sequence were synthesized, based on observations from the crystal structure (Figure 2). First, the side chain of D-Arg[1] adopts a high-energy, eclipsed conformation. Further, this conformation places the N-terminal amine in close proximity (3.8 Å) to the positively charged nitrogen of the guanidino group of the side chain, resulting in an additional energetic penalty via an unfavorable electrostatic interaction. Since the guanidino group makes polar interactions with the side chain of D189 as well as backbone carbonyls of G218 and A190 (10), a positively charged moiety is desirable on this side chain. The N-terminal amine, however, does not make any contacts with thrombin, and so removal of this group (compound **1**) would be expected to eliminate the unfavorable electrostatic clash without eliminating any favorable interactions and thus result in improved potency. The length of the side chain was also investigated (**2**–**5**) to potentially eliminate the eclipsed conformation of the side chain. Additionally, replacement of the guanidino group with an amino group on the side chain was explored (**6**–**8**), while maintaining the same linker length between the backbone carbonyl carbon of the residue and the positively charged moiety on the end of the side chain (guanidino or amino). Finally, one conformationally restricted analog was synthesized (**9**), in an attempt to lock in the eclipsed conformation observed in the X-ray structure. Analytical data for all nine compounds along with lead compound FM 19 are displayed in Table 1. Thrombin inhibition data for all nine compounds as well as lead compound FM 19 are shown in Table 2. Structures for all replacements are shown in Figure 3.

Compound **1** (5-guanidinopentanoic acid) differs from lead compound FM 19 only in the removal of the N-terminal amine. Thus, its side chain guanidino group maintains the same distance from the backbone carbonyl carbon as in the D-Arg[1] of FM 19. As seen in Table 2, compound **1** shows considerable improvement over lead compound FM 19 (over sixfold increase in potency). Thus, removal of the N-terminal amine, and consequent elimination of the adverse electrostatic interaction seen in the X-ray structure of FM 19 bound to thrombin, does, as predicted, lead to an increase in potency.

Table 1: Analytical data: D-arginine replacements in the FM 19 sequence (X-Oic-Pro-D-Ala-Phe (*p*-Me)-NH_2)

Compound	D-Arg replacement (X)	Expected [M + H]	Found [M + H]	Analytical HPLC retention time (min)
FM 19	D-Arg	654.4	654.4	26.5
1	5-Guanidinopentanoic acid	639.4	639.4	32.6
2	Guanidinoglycine	597.3	597.3	30.1
3	3-Guanidinopropionic acid	611.4	611.2	30.1
4	4-Guanidinobutyric acid	625.4	625.3	31.4
5	6-Guanidinohexanoic acid	653.4	653.5	33.9
6	5-Aminopentanoic acid	597.4	597.2	30.6
7	6-Aminohexanoic acid	611.4	611.4	31.6
8	7-Aminoheptanoic acid	625.4	625.3	33.5
9	*p*-Guanidinobenzoic acid	659.4	659.3	31.6

Table 2: Thrombin inhibition potencies of D-arginine replacements in the FM 19 sequence (X-Oic-Pro-D-Ala-Phe(*p*-Me)-NH_2)

Compound	D-Arg replacement (X)	IC_{50} (μM)
FM 19	D-Arg	4.4 ± 1.3
1	5-Guanidinopentanoic acid	0.66 ± 0.20
2	Guanidinoglycine	36.8 ± 9.4
3	3-Guanidinopropionic acid	6.5 ± 0.6
4	4-Guanidinobutyric acid	8.4 ± 2.0
5	6-Guanidinohexanoic acid	25.6 ± 6.8
6	5-Aminopentanoic acid	241 ± 41
7	6-Aminohexanoic acid	130 ± 31
8	7-Aminoheptanoic acid	498 ± 14
9	*p*-Guanidinobenzoic acid	0.57 ± 0.12

Compounds **2**–**5** explore the effects of a different linker length, while still containing a guanidino group on the side chain and eliminating the N-terminal amine. Compounds **2** (guanidinoglycine), **3** (3-guanidinopropionic acid) and **4** (4-guanidinobutyric acid) incorporate a shorter linker, while compound **5** (6-guanidinohexanoic acid) incorporates a longer one, compared to FM 19. All four analogs show reduced potency compared to compound **1**, with the shortest (**2**) and longest (**5**), side chains displaying lower potency than the intermediate analogs **3** and **4**. Although compound **4** is over 10-fold less potent than compound **1**, it might be expected to be better, since its shorter side chain would allow interaction with thrombin without the need for an eclipsed conformation. Presumably, the guanidino group is not positioned correctly in **4** to make optimal interactions. Compound **3**, with a shorter, less flexible side chain than **4** would be expected to suffer less of an entropy loss upon binding. However, **3** shows similar inhibitor potency to **4**, presumably because the side chain of **3** is too short to perfectly position the guanidino as in **1**, similar to **4**. Thus, even though it adopts a strained eclipsed conformation, it appears as if compound **1** has the optimal side-chain length of six atoms between the carbonyl carbon and positively charged guanidino group on the end of the side chain to provide favorable interaction with the thrombin active site.

Next, replacement of the guanidino group with an amino group was investigated, while maintaining the same linker length between the backbone carbonyl carbon and the positively charged moiety. As seen in Table 2, compound **3** (3-guanidinopropionic acid) is more than 30 times more potent than its amino-containing counterpart, compound **6** (5-aminopentanoic acid). Similarly, compound **4** (4-guanidinobutyric acid) is more than 15 times more potent than compound **7** (6-aminohexanoic acid). Finally, compound **1** (5-guanidinopentanoic acid) is over 750 times more potent than compound **8** (7-aminoheptanoic acid). The comparison of these pairs of compounds suggests that the guanidino group, and not merely a cationic moiety, is important for potency. The difference in potency seen in the last pair of compounds (**1** versus **8**) is much larger than that found in the other pairs, and might be attributed to entropic effects, since these compounds have the longest side chain length. It is possible that the entropic penalty encountered with constricting compound **8** into a proper binding orientation is too large so that the enthalpy gain upon binding is not enough to produce a similarly potent compound. It has been suggested that delocalization of the positive charge over more than one atom in a guanidino group allows for greater polar contacts with the active site D189 than a point charge on an amino group (15). The results for compounds **6**–**8** support this suggestion.

Finally, compound **9** (*p*-guanidinobenzoic acid) was synthesized in an attempt to lock in the eclipsed conformation that D-Arg^1 of FM 19 adopts upon binding to thrombin's active site. Like compound **1**, compound **9** contains no N-terminal amine and has an equivalent side chain length, but in addition is conformationally constrained to adopt an eclipsed orientation. Accordingly, compound **9** might be expected to be more potent than compound **1**. Instead, this compound is equipotent to compound **1**. A possible explanation could be that, while the D-Arg^1 side chain in the FM 19 crystal structure is not completely planar, the *p*-guanidinobenzoic acid side chain of compound **9** is. This could cause the guanidino group of compound **9** to point into a slightly different, less optimal position, resulting in a lower than expected potency. It is also possible that steric effects because of the remainder of the phenyl ring may lower binding affinity for thrombin.

Conclusions

Two compounds, **1** and **9**, have been synthesized that show a significant increase in human α-thrombin inhibition over the previously reported lead compound FM 19. Both compounds eliminate the N-terminal amine, which improves potency as predicted from the crystal structure. Both compounds also contain a highly basic guanidino group that increases potency of this class of

compounds. Compound **9** also adds conformational restriction to the modified side chain to lock in the eclipsed conformation. Further studies are underway that will explore additional conformational restriction on this side chain, as well as additional guanidino modifications that maintain basicity as well as delocalization of positive charge.

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Note

[a]v.5; GraphPad Software, Inc., La Jolla, CA, USA.