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Design and Synthesis of Thrombin Receptor-derived Nonpeptide Mimetics Utilizing a Piperazine Scaffold

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Abstract—Focal thrombus formation and vasoconstriction serve to defend vessels when vascular damage occurs, but may be detrimental when an atherosclerotic plaque is disrupted. Recently, the identification of the platelet thrombin receptor opened a new area in the development of agents that may selectively inhibit the effects of thrombin on cells, without affecting fibrin formation. In this regard, we have synthesized a number of 1,4-disubstituted piperazines which are designed to be analogues of thrombin receptor activating peptides (TRAP) and carry the pharmacophoric features of Phe and Arg residues present in the active pentapeptide SFLLR. These compounds were tested in the rat aorta relaxation assay and in platelet aggregation studies and their biological activity was consistent with a direct action on thrombin receptor. Furthermore, the structure–activity relationships confirmed the importance of Phe and Arg for receptor activation and the molecular modeling revealed an intriguing relationship between their amphipathic similarity with SFLLR and their biological activity.

Introduction

Thrombin is a serine protease that in addition to being a key enzyme in thrombus formation, can activate a variety of cells such as platelets, endothelial cells, fibroblasts and vascular smooth muscle.[1–3] The identification of the platelet thrombin receptor and the demonstration that is a member of the super-family of G-protein-coupled receptors[4,5] opened a new area in the development of agents that may selectively inhibit the effects of thrombin on cells, without affecting fibrin formation. The activation mechanism of target cells by thrombin involves a proteolytic cleavage of the extracellular N-terminal bond between Arg_{41} and Ser_{42} of the thrombin receptor. The newly generated N-terminus unmasked by thrombin-induced cleavage serves as a tethered ligand[6] which binds intramolecularly to effect receptor activation.[7,8] In support of this model, synthetic peptides (thrombin receptor activating peptides (TRAP)) corresponding to at least the five residues of the new N-terminus ($S_{42}FLLR_{45}$) are effective in mimicking the action of thrombin.[9–12] Structure–activity relationships (SAR) and alanine scan experiments have indicated that Phe_{43} and Arg_{45} are the most important amino acids of the receptor-derived peptide SFLLR for its activity in smooth muscle[13,14] and for its ability to aggregate platelets.[9,10] We have recently shown, using cyclic TRAP, the importance of the Phe/Arg relative conformation for activity in a smooth muscle contractile assay.[14] Considering the above information and based on previous NMR and SAR experiments,[15–17] we proceeded in the synthesis of non-peptide analogues of the minimal SFLLR sequence using piperazine as the template to hold groups that can bear the pharmacophoric features of Phe and Arg (Scheme 1). The synthesized compounds were initially tested for their biological activity in the rat aorta relaxation assay,[12,13] and three representative compounds with low, medium and high biological activity (compounds **11**, **10** and **13**, respectively) were further tested to evaluate their ability to affect in vitro the aggregation of human platelets induced by either thrombin or the linear thrombin receptor activating amide SFLLR-NH_2. This peptidomimetic research is

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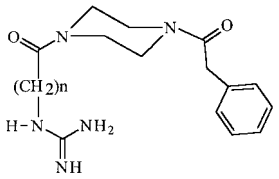


n = 1, 2, 3, 5

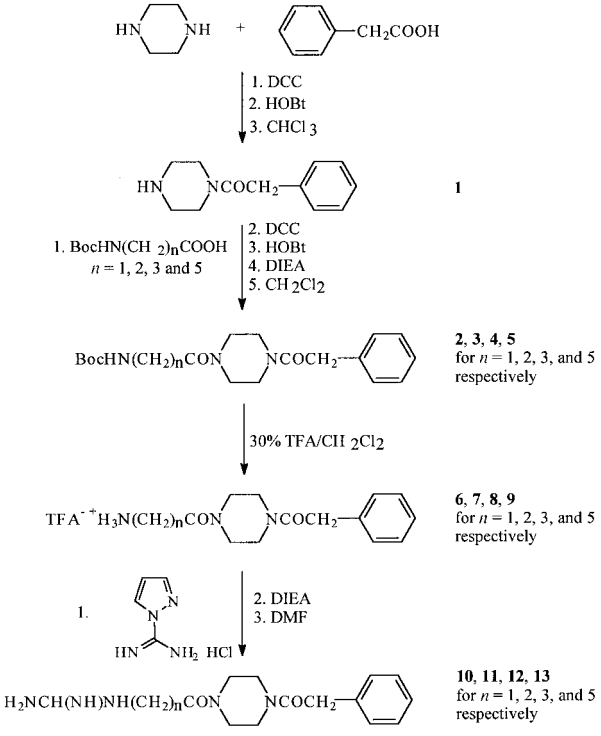
Scheme 1. Nonpeptide TRAP derived mimetics incorporating piperazine.

growing rapidly in an effort to improve the metabolic stability, duration of action and bioavailability of the endogenous peptides.[16]

Results

Chemistry

The general methodology for the synthesis of 1-4 disubstituted piperazine derivatives is depicted in Scheme 2. 1-Phenylacetyl-piperazine **1** was synthesized by coupling piperazine and phenylacetic acid with DCC and HOBt as coupling reagents. Boc-protected amino acids (glycine, β-alanine, γ-aminobutyric and ε-aminohexanoic acid) were then incorporated separately in **1** aided by the use of DCC and HOBt under basic conditions (DIEA). The Boc-protecting group was preferred over the Fmoc group because of the problems that the latter causes through its removal with piperidine during the cleavage step. Boc-deprotection of each Boc-derivative



Scheme 2. Synthetic procedure for 1,4-disubstituted piperazine derivatives.

(**2**, **3**, **4**, and **5**) was accomplished with trifluoroacetic acid giving the free-amine salts **6**, **7**, **8**, and **9**. Guanylation of these amines took place according to the procedure described by Bernatowicz.[18] For example, the reaction of pyrazole with cyanamide in *p*-dioxane gave 1*H*-pyrazole-1-carboxamide hydrochloride in high yield. This compound served as a guanylation reagent which reacted with the previous amines to give **10**, **11**, **12**, and **13** as final products.

Rat aorta relaxation assay

Of the compounds synthesized, compound **13**, with an EC_{50} of about 150 μM (Fig. 2) was clearly the most active, followed by compound **10**. Compounds **7**, **8**, and **9** showed minimal activity; and compounds **6**, **11**, and **12** appeared to be devoid of activity. When tested at concentrations higher than 500 μM, compounds **6**, **7**, **8**, **9**, **11**, and **12** appeared to show toxicity, in that the subsequent contractile responsiveness of the tissues to phenylephrine was compromised. This was not the case for compounds **10** and **13**.

Because thrombin induces endothelium-dependent, nitric-oxide-mediated vascular relaxations through the activation of the tethered ligand receptor on endothelial cells, the mechanism of vasorelaxation induced by the most active compound **13**, was further examined. As shown in Figure 1, the relaxant activity of compound **13** was, like that of acetylcholine, dependent upon the presence of an intact endothelium (Tracing A) and it was not observed after the endothelium had been denuded, although the ability of the same preparation to relax in response to isoproterenol via an endothelium-independent mechanism remained intact (Tracing C). Moreover, in the same endothelium-containing preparation, the relaxant activity of compound **13** was sustained, while that of the thrombin receptor activating pentapeptide, P5-NH_2 was rather transient (Tracings A

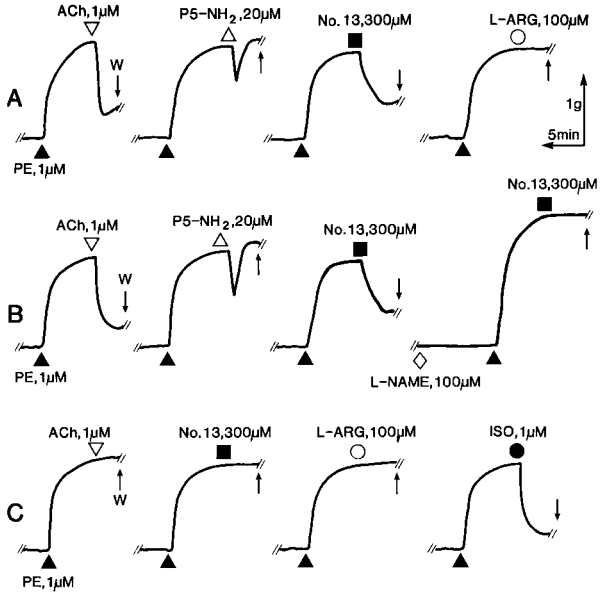


Figure 1. Relaxant action of compound **13** (300 μM) compared with P5-NH_2 and Ach.

and B). As shown previously with acetylcholine, thrombin and the thrombin receptor activating peptide (P5-NH_2), the compound **13**-induced vasorelaxation was completely blocked by the nitric oxide synthase inhibitor, L-NAME (Tracing B, and data not shown). Finally, the relaxant activity of compound **13** could not be attributed to the partial metabolism of its guanidino side-chain to nitric oxide, since L-arginine itself, which could be similarly metabolized, failed to cause a relaxant response (Tracing A).

Platelet aggregation studies

Incubation of platelet-rich plasma with varying concentrations (0–4.0 mM) of the nonpeptide TRAP analogues (compounds **10**, **11**, and **13**) failed to produce platelet aggregation, indicating that these molecules were devoid from any thrombin-like agonistic activity on human platelets. In contrast, when platelet-rich plasma was preincubated with each compound for 4 min prior to the addition of thrombin or SFLLR-NH_2, their ability to behave like both thrombin and SFLLR-NH_2 antagonists was brought to light. As shown in Figure 3(A), compound **10** was able to significantly inhibit platelet aggregation when a low concentration of thrombin ($\leqslant$0.2 NIU/mL) was used, but failed to inhibit platelet aggregation when higher concentrations ($>$0.3 NIU/mL) of thrombin were used. Compound **11** inhibited thrombin-induced platelet aggregation only at high concentration (2.0 mM, Fig. 3(B)). Interestingly, compound **13** was capable to produce a significant and dose-dependent inhibition of the thrombin-induced platelet aggregation even when relatively low concentrations of this compound (0.25 mM) were utilized (Fig. 3(C)).

The IC_{50} for compound **10** was 0.796 mM for thrombin concentration 0.2 NIU/mL and 1.833 mM in the case of thrombin range 0.25–0.3 NIU/mL. For compound **11** the IC_{50} was 0.867 mM when thrombin concentration was 0.2 NIU/mL whereas for thrombin range 0.25–0.3 NIU/mL was 0.895 mM. For compound **13** the IC_{50} was found to be 0.466 mM for thrombin concentration 0.2 NIU/mL and 0.481 for thrombin range 0.25–0.3 NIU/mL. Finally, all three compounds were shown to significantly inhibit the SFLLR-NH_2-induced (Fig. 4), but not the collagen-induced platelet aggregation.

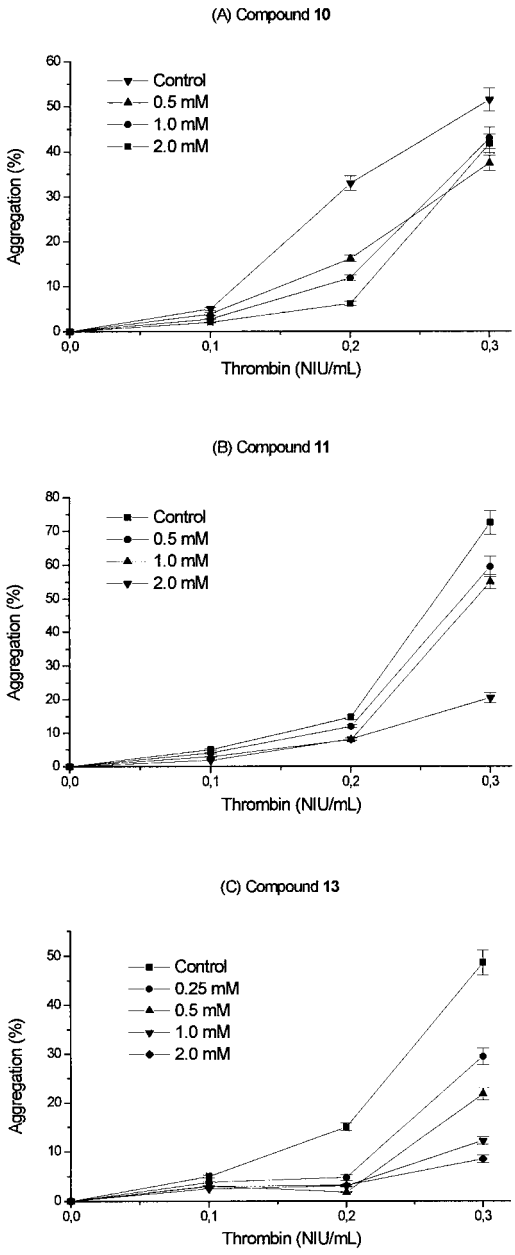


Figure 3. Effect of nonpeptide thrombin mimetics (compounds **10**, **11**, and **13**) on inhibition of thrombin-induced platelet aggregation in human PRP. These data are representative of nine experiments giving similar results ($p < 0.001$).

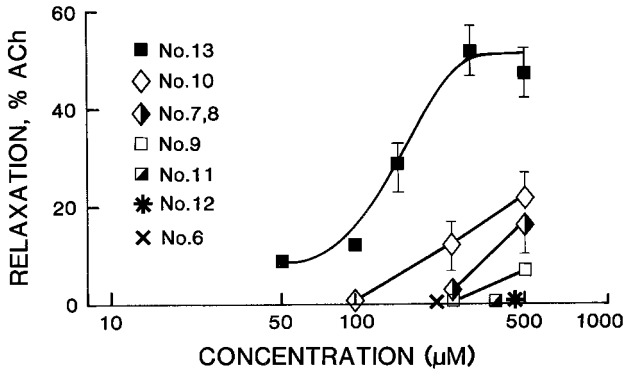


Figure 2. Concentration-effect curves for compounds **8**–**13**.

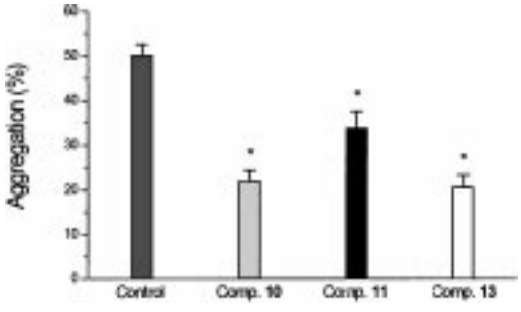


Figure 4. Effect of nonpeptide thrombin mimetics (compounds **10**, **11**, and **13**) on inhibition of SFLLR-NH_2 (1 μM)-induced platelet aggregation in human PRP. Data are expressed, as mean ± SEM of four experiments (*$p < 0.0001$ versus control).

Molecular Modeling

Theoretical calculations were performed on a Silicon Graphics 4-D/35 model using the Quanta 3.3 version of molecular simulation incorporated (MSI) program. The constructed structures of all eight molecules were first minimized. Constraints on the minimized structures were imposed in order to achieve a close proximity between Phe and Arg (or -NH_2 moieties). The superimposition of pairs of structures was performed using rigid body fit to target method of molecular similarity software. Rigid body fitting translates and rotates working structures (**6**–**13**) to minimize the RMS (root mean square) of the fit to the target structure (SFLLR). Details of this approach was given in our previous publications.[14,19]

Discussion

The identification of the human thrombin receptor has generated significant interest in the development of receptor antagonists that can inhibit the effects of thrombin on cells without affecting its enzymatic activity on fibrin formation. Synthetic peptides of which the first five residues contain the tethered ligand domain SFLLR have been found to be capable of causing the cellular effects of thrombin. It is well established that the introduction of non-peptide molecules into peptides can dramatically affect receptor binding affinity.[20,21] The design of non-peptide mimetics of TRAP, such as the ones described here, has been based on the current knowledge about the interactions of the tethered ligand and the thrombin receptor.[14–16] The fact that the cyclic conformer of SFLLR observed by NMR is probably the bioactive species, along with the observation that certain cyclic derivatives are biologically active[22] prompted us to design molecules that mimic the cyclic conformation adopted by SFLLR. In this conformation the Phe and Arg residues are in a close proximity on the same side of the cyclic ring. Replacing the aliphatic residues of the active peptide core with piperazine would be one way of stabilizing and testing compounds able to adopt the above cyclic conformation. Indeed, piperazine has been widely used as a template in medicinal chemistry[23] and this and other templates are currently under investigation in our laboratory for their ability to maintain the pharmacophoric features of Phe and Arg in a suitable conformation. Such mimetics could be valuable in the development of a lead structure useful in drug optimization. The design of mimetics was assembled according to Scheme 1. The pharmacophoric characteristic of Phe was incorporated by coupling phenylacetic acid to piperazine, while the pharmacophoric feature of Arg was built in through a variety of guanidino linear chains attached to the other end of piperazine. The different chain lengths could give us information about the hydrophobic core between the guanidino and phenyl groups through structure–activity relationship studies.

Most of the compounds showed moderate activity in the rat aorta relaxation assay. Compounds **7**–**9** had weaker activity relative to **10** and **13** indicating that the guanidino group is more essential than the amino group for activation of the receptor. In addition, compound **13** showed the best NO-mediated endothelial cell-dependent relaxation among the compounds tested (Fig. 2) indicating that the alkyl guanidine chain length may also be important for optimizing the activity. Because the rat aorta relaxation assay is a complex system the possibility could not be denied that the TRAP analogues tested here produced vasorelaxation by mechanism(s) not involving the thrombin receptor. Indeed, other 1,4-disubstituted piperazines have been previously shown to stimulate a variety of receptors, including the receptor for the platelet-activating factor[24,25] and the adhesion receptor.[26]

The effects of thrombin on platelets are mediated by thrombin receptors. Since in our experience and that of other investigators[27] rat platelets are poorly reactive to thrombin and to SFLLR-NH_2, we chose human platelets to evaluate the ability of three representative compounds (compounds **10**, **11**, and **13**) to induce platelet aggregation or inhibit the thrombin- or SFLLR-NH_2-induced platelet aggregation. Although none of these compounds showed any agonistic activity when incubated with PRP, they were all able to significantly inhibit the thrombin- or SFLLR-NH_2-induced platelet aggregation. Of note, compound **13** was clearly the most effective, as it was the case in the rat aorta relaxation assay. Taking into consideration that (a) the effects of thrombin on platelets are mediated through its specific receptor, (b) SFLLR-NH_2 directly deals with thrombin receptor, and (c) the compounds had no effect on collagen-induced platelet aggregation, we may come to the conclusion that the compounds tested with the platelet aggregation studies exert their biological activity through specific interactions with the thrombin receptor rather than by inhibiting thrombin's enzymatic activity or stimulating other type of receptor(s).[28]

Several tentative reasons to explain the difference between the thrombin-like agonistic effect of these compounds in the rat aorta relaxation assay and the anti-thrombin effect in the plasma aggregation assay can be proposed here. Firstly, different types of thrombin receptor may exist in different species. In this regard, human but not rat and dog platelets are activated by SFLLR peptides.[27] Secondly, different types of thrombin receptor(s) and different activating pathways may be stimulated in platelets and in endothelial cells. Indeed, similar results to our findings have been reported for Mpa-peptides.[29,30] Nevertheless, the existence of a molecule that could both inhibit platelet aggregation and augment vasorelaxation would be of paramount clinical significance and it could potentially be used to prevent vascular reocclusion after thrombolytic therapy of acute myocardial infarction or after coronary artery angioplasty.[31]

To further examine the stereoelectronic factors that determine the biological activity of these compounds the experimental data were coupled with theoretical calculations. As was mentioned above, we have recently proposed a cyclic model shown in Figure 5 for SFLLR based on a combination of results obtained from biological assays, NMR NOE spectroscopy and molecular modeling.[14,15,17] The main feature of this model is that

Phe and Arg aminoacid residues are in close proximity and their side chains are characterized by reduced mobility. Interactions between Arg and Phe side chains have also been observed in the crystal structures of proteins.[21] Synthesized structures **6**–**13** were constructed and minimized using distance constrains in order to achieve a spatial vicinity between Phe and Arg (or -NH_2 terminal) moieties. The two sets of compounds (**6**–**9** and **10**–**13**) were overlaid with SFLLR in order to achieve best matching. Their superimposition was based on the following group matches: (i) Phe of SFLLR with Phe of compounds **6**–**13**, (ii) Arg of SFLLR and Arg of **10**–**13** or -NH_2 terminal group of **6**–**9**. The best overlay between the two sets of the synthesized molecules was achieved with compounds **9** of the first set (Fig. 6) and **13** of the second set (Fig. 7). Both compounds have the longer alkyl chain and differ only at one terminal end. Analogue **9** possess an amino group and **13** a guanidino one. The RMS values for the superimposition between SFLLR and **9** or **13** were 0.83 and 0.19, respectively. It appears that the superimposition between SFLLR and the most active synthetic analogue **13** was better than SFLLR and **9**. These results point out the importance of the Arg aminoacid in the synthesized peptidomimetic analogues. Arginine contains more polar atoms (three nitrogens) versus one nitrogen that is contained in the aminoacid lysine of **9**. This polar similarity between SFLLR and analogue **13** may account for its better biological profile when compared with **9**. Comparison between compound **13** and the other compounds of the same data set (**10**–**12**) shows that this differs only in the length of the alkyl chain (Fig. 8). Compound **13** has the longest alkyl chain. This gives an advantage to this molecule to maximize its hydrophobic interactions with leucine of SFLLR. This close proximity between the long alkyl chain of compound **13** and leucine of SFLLR may explain its higher biological activity vis a vis compounds **10**–**12**. In general, compound **13** is the most biologically active compound because: (i) it contains both Phe and Arg, the key aminoacid residues present in SFLLR; (ii) possess a long alkyl chain that allows it for a close proximity with the hydrophobic Leu residue of SFLLR. Therefore, the emerging picture for these comparisons is that amphipathic interactions are responsible for optimum activity in the studied peptidomimetic analogues. Synthesis of new analogues will lead to more accurate determination of the key stereoelectronic factors that determine their biological activity.

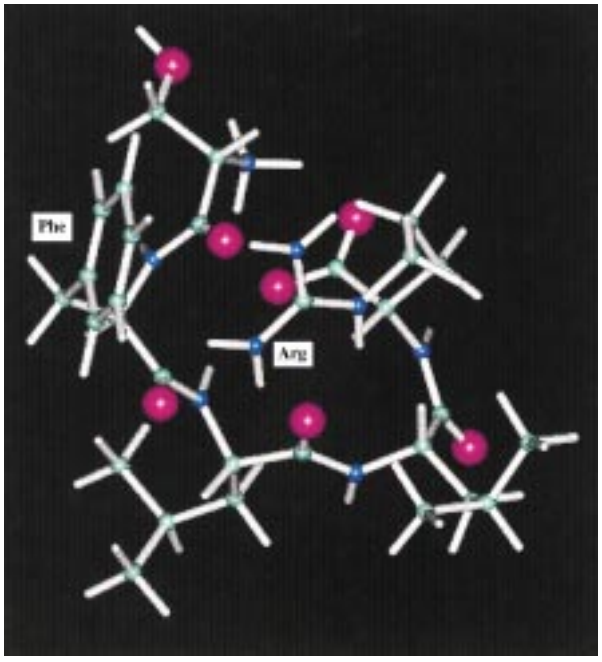


Figure 5. Proposed model of SFLLR generated by imposing distance geometry constraints between Phe and Arg aminoacids and use of molecular dynamics.

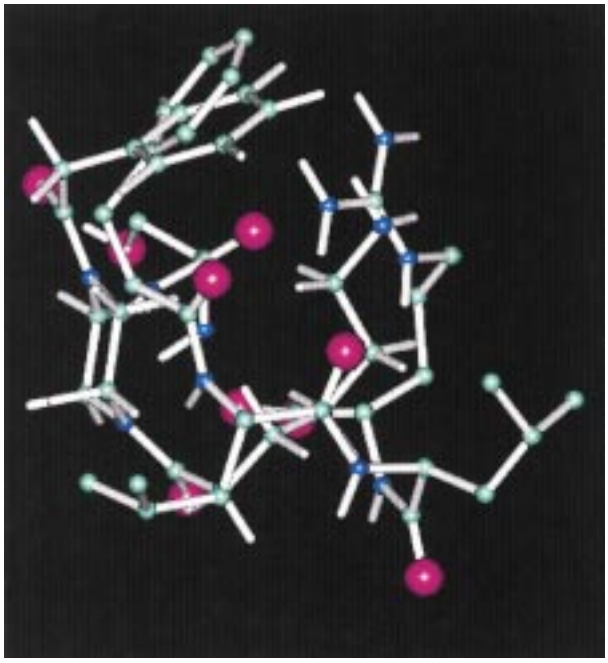


Figure 6. Superimposition of the synthetic analogue **9** with SFLLR using body fit to target method.

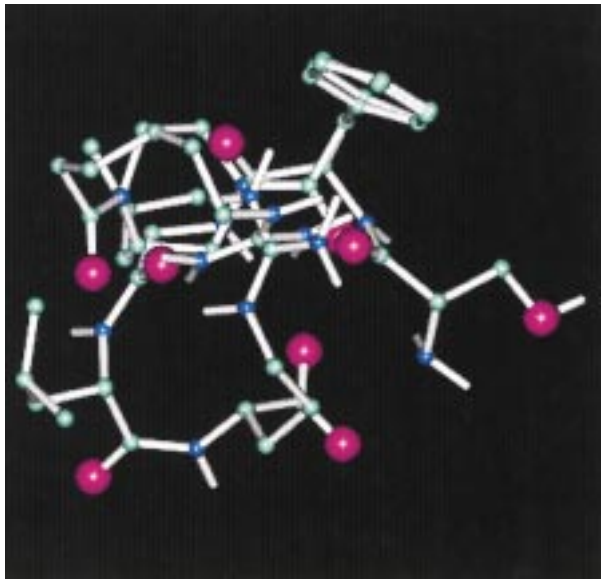


Figure 7. Superimposition of the synthetic analogue **13** with SFLLR using rigid body fit to target method.

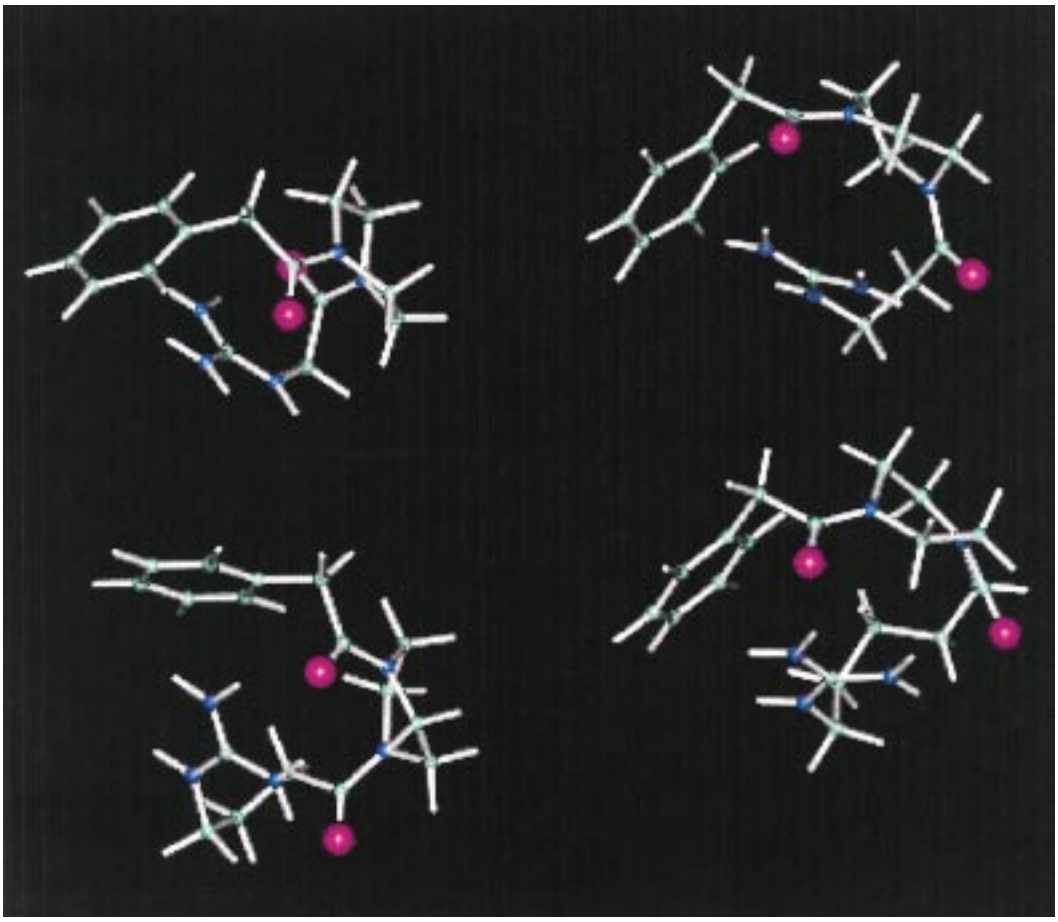


Figure 8. Low energy conformers generated by imposing distance geometry constraints between Phe and Arg aminoacids of synthetic compounds **10** (top, left) **11** (top, right), **12** (bottom, left), and **13** (bottom, right).

Conclusion

We have described the synthesis of novel low weight 1,4-disubstituted piperazines derived from TRAP that interact with the thrombin receptor. Synthesis of such novel peptidomimetic analogues aims (i) to investigate the role of piperazine as a template in drug design and (ii) to examine the interactions between Phe and Arg residues. Among the synthesized compounds, **10** and **13** were found to be the most active in the rat aorta relaxation assay and in platelet aggregation studies, confirming our proposed model for the interaction between TRAP and the receptor. Furthermore, the significance of Arg residue of the tethered ligand for activity is once more indicated. These are lead compounds for further development. It is anticipated that modification of **13** will provide us with better mimetics. For example, utilization of piperazine constraints, substitution of phenyl group or even employment of different templates could give us more information in our ongoing effort to develop peptide mimetics for drug therapy.

Experimental

Chemistry

The ^{1}H NMR spectra were recorded on a Bruker ACF-300 spectrometer operating at 7.05 T. All NMR spectra were obtained in $CDCl_3$ or DMSO-d_6. All chemical shifts were referenced to tetramethylsilane (TMS). The accurate M^+ of the synthesized compounds were obtained on a Kratos Profile HV-3 mass spectrometer operating with mass range among 0–1200 amu at 4 kV and resolution variable from nominal mass (600) to accurate mass (10,000). Most of the compounds were purified by flash chromatography using Merck silica gel 60 (230–400 mesh). Thin-layer chromatography (TLC) was performed using Merck pre-coated silica gel 60 F-254 glass plates. Visualization was accomplished by using a combination of UV-lamp and ninhydrin spray. All synthetic reagents were purchased either from the Aldrich Chemical Company or the Sigma Chemical Company and were used without purification. Solvents were analytical reagent grade or better and were used as supplied. All air- and moisture-sensitive reactions were performed under nitrogen. A Buchi RE 111 rotavapor was utilized for the removal of the solvents in vacuo. Moisture from the hydroscopic arginine derivatives was removed using a freeze-drying pump. Melting point determinations were performed on a Buchi 530 melting point apparatus.

1-(Phenylacetyl)piperazine (1). Phenylacetic acid (1.65 g, 12.2 mmol) and HOBt (1.65 g, 12.2 mmol) were suspended in $CHCl_3$ (25 mL). DCC (2.51 g, 12.2 mmol) was

then added followed by DMF (3 mL). The mixture was stirred for ~30 min at room temperature to give a white suspension. The mixture was transferred slowly to a pre-cold solution of piperazine (5.24 g, 60.9 mmol) in 60 mL $CHCl_3$. The reaction was stirred for 2 h at room temperature and then the white suspension (DCU) was filtered and the filtrate was acidified with 2 M HCl (3×30 mL). The HCl extracts (120 mL) were basified with 2 M NaOH, extracted with $CHCl_3$ (4×40 mL), dried over anhydrous Na_2SO_4 and reduced in vacuo to give 2.27 g (11.1 mmol, 91%) of a pale-green liquid as a crude product. Flash chromatography (7% MeOH:$CHCl_3$) yielded 1.87 g (9.2 mmol, 75%) of a colorless oil. R_f 0.38 (ButOH:H_2O:AcOH, 4:1:1), ^{1}H NMR ($CDCl_3$): δ 1.8 (s, 1H), 2.6 (t, 2H), 2.8 (t, 2H), 3.4 (t, 2H), 3.6 (t, 2H), 3.7 (s, 2H), 7.2–7.3 (m, 5H), MS (nominal): 204 (M^+),113 ($M^+-C_6H_5CH_2^{\cdot}$), MS (accurate): M^+ calcd: 204.1263, M^+ found: 204.1267.

1-Phenylacetyl-4-[2-(*tert*-butoxycarbonylamino)acetyl]-piperazine (2). 1-(Phenylacetyl)piperazine (400 mg, 1.9 mmol), Boc-Gly-OH (2.0 mmol), HOBt (284 mg, 2.1 mmol) and DIEA (1 mL) were dissolved in 15 mL CH_2Cl_2. The mixture was cooled to 0 °C and DCC (433 mg, 2.1 mmol) was added. The reaction was stirred at this temperature for ~30 min and then at room temperature. The reaction was monitored by TLC (20% MeOH:$CHCl_3$) and showed completion after ~18 h. The suspension (DCU) was filtered and washed with 25 mL CH_2Cl_2. The filtrate was washed with 15% citric acid (2×20 mL), satd $NaHCO_3$ solution (2×20 mL) and H_2O (2×20 mL), dried over Na_2SO_4 and concentrated in vacuo to give an orange-yellow solid as a crude product. Flash chromatography (2% MeOH:$CHCl_3$) yielded a waxy white solid (397 mg, 1.1 mmol, 58%). R_f 0.79 (30% MeOH:$CHCl_3$), ^{1}H NMR ($CDCl_3$): δ 1.4 (s, 9H), 3.4 (d, 2H), 3.1–3.9 (m, 8H), 3.7 (s, 2H), 5.4 (br s, 1H), 7.2–7.3 (m, 5H), MS (nominal): 361 (M^+), 288 ($M^+-(CH_3)_3CO^{\cdot}$), MS (accurate): M^+ calcd: 361.2002, M^+ found: 361.2009.

1-Phenylacetyl-4-[3-(*tert*-butoxycarbonylamino)propionyl]-piperazine (3). The same procedure described as above for **2** was followed except that Boc-β-Ala-OH was used instead of Boc-Gly-OH. The product was obtained pure in 59% yield, after flash chromatography (2% MeOH:$CHCl_3$). R_f 0.80 (30% MeOH:$CHCl_3$), ^{1}H NMR ($CDCl_3$): δ 1.4 (s, 9H), 2.5 (t, 2H), 3.4 (m, 2H), 3.1–3.6 (m, 8H), 3.7 (s, 2H), 5.2 (br s, 1H), 7.2–7.3 (m, 5H), MS (nominal): 375 (M^+), 302 ($M^+-(CH_3)_3CO^{\cdot}$), MS (accurate): M^+ calcd: 375.2158, M^+ found: 375.2157.

1-Phenylacetyl-4-[4-(*tert*-butoxycarbonylamino)butyryl]-piperazine (4). The same procedure described as above for **2** was followed except Boc-γ-Abu-OH was used instead of Boc-Gly-OH. The product was obtained pure in 69% yield, after flash chromatography (2% MeOH:$CHCl_3$). R_f 0.81 (30% MeOH:$CHCl_3$), ^{1}H NMR ($CDCl_3$): δ 1.4 (s, 9H), 1.8 (t, 2H), 2.4 (m, 2H), 3.1 (m, 2H), 3.1–3.6 (m, 8H), 3.7 (s, 2H), 4.7 (br s, 1H), 7.2–7.3 (m, 5H), MS (nominal): 389 (M+), 316 ($M^+-(CH_3)_3CO^{\cdot}$), MS (accurate): M^+ calcd: 389.2315, M^+ found: 389.2324.

1-Phenylacetyl-4-[6-(*tert*-butoxycarbonylamino)hexanoyl]-piperazine (5). The procedure described above for **2** was followed except that Boc-ε-Ahx-OH was used instead of Boc-Gly-OH. The product was obtained pure in 64% yield, after flash chromatography (2% MeOH:$CHCl_3$). R_f 0.83 (30% MeOH:$CHCl_3$), ^{1}H NMR ($CDCl_3$): δ 1.3 (t, 2H), 1.4 (s, 9H), 1.5 (m, 2H), 1.6 (m, 2H), 2.3 (m, 2H), 3.1 (m, 2H), 3.2–3.6 (m, 8H), 3.7 (s, 2H), 4.6 (br s, 1H), 7.2–7.3 (m, 5H), MS (nominal): 417 (M^+), 344 ($M^+-(CH_3)_3CO^{\cdot}$), MS (accurate): M^+ calcd: 417.2627, M^+ found: 417.2619.

General procedure for Boc-deprotection with trifluoroacetic acid. Each of the Boc-derivatives (1.0 mmol) was dissolved in 3 mL of a 30% TFA:CH_2Cl_2 solution and the mixture was stirred for 1 h. The solution was evaporated to dryness in vacuo and the residue was triturated with dry ether giving a yellowish oil (0.9 mmol, 90%).

1-Phenylacetyl-4-(2-aminoacetyl)piperazine trifluoroacetic salt (6). R_f 0.22 (30% MeOH:$CHCl_3$), ^{1}H NMR (DMSO-d_6): δ 3.2–3.7 (m, 8H), 3.5 (m, 2H), 3.8 (s, 2H), 7.2–7.3 (m, 5H), 8 (br s, 3H), MS (nominal): 261 (M^+−TFA), MS (accurate): (M^+−TFA) calcd: 261.1478, (M^+−TFA) found: 261.1474.

1-Phenylacetyl-4-(3-aminopropionyl)piperazine trifluoroacetic salt (7). R_f 0.24 (30% MeOH:$CHCl_3$), ^{1}H NMR ($CDCl_3$): δ 2.7 (br s, 2H), 3.2–3.6 (m, 8H), 3.4 (m, 2H), 3.7 (s, 2H), 7.2–7.3 (m, 5H), 8 (br s, 3H), MS (nominal): 275 (M^+−TFA), MS (accurate): (M^+−TFA) calcd: 275.1634, (M^+−TFA) found: 275.1639.

1-Phenylacetyl-4-(4-aminobutyryl)piperazine trifluoroacetic salt (8). R_f 0.29 (30% MeOH:$CHCl_3$), ^{1}H NMR ($CDCl_3$): δ 1.9 (br s, 2H), 2.4 (br s, 2H), 3.0–3.6 (m, 8H), 3.4 (br s, 2H), 3.7 (s, 2H), 7.2–7.3 (m, 5H), 8 (br s, 3H), MS (nominal): 289 (M^+−TFA), MS (accurate): (M^+−TFA) calcd: 289.1790, (M^+−TFA) found: 289.1795.

1-Phenylacetyl-4-(6-aminohexanoyl)piperazine trifluoroacetic salt (9). R_f 0.36 (30% MeOH:$CHCl_3$), ^{1}H NMR ($CDCl_3$): δ 1.3 (t, 2H), 1.5 (m, 2H), 1.6 (m, 2H), 2.3 (m, 2H), 2.9–3.6 (m, 8H), 3.4 (br s, 2H), 3.7 (s, 2H), 7.2–7.3 (m, 5H), 8 (br s, 3H), MS (nominal): 317 (M^+−TFA), MS (accurate): (M^+−TFA) calcd: 317.2103, (M^+−TFA) found: 317.2108.

General procedure for the guanylation of primary amine derivatives. TFA-salt (0.7 mmol), 1*H*-pyrazole-1-carboxamidine hydrochloride (175 mg, 1.2 mmol) and DIEA (0.7 mL) were dissolved in 2 mL DMF giving a green-brown liquid. The mixture was stirred under nitrogen for ~24 h at room temperature. Then, ether (15 mL) was added and the product appeared as gel at the bottom of the flask, while the supernatant liquid was decanted. The crude product was triturated with acetone:Et_2O and finally the remaining moisture was removed in a freeze-drying pump to give a grey solid which was recrystallized from MeOH:Acetone:Et_2O to give pure product (0.35 mmol, 50%).

1-Phenylacetyl-4-(2-guanidoacetyl)piperazine (10). Melting point: 190–191 °C, R_f 0.40 (ButOH:H_2O:AcOH, 4:1:1), ^{1}H NMR (DMSO-d_6): δ 3.3–3.5 (m, 8H), 5.4 (m, 2H), 3.7 (s, 2H), 7.2–7.3 (m, 5H), 7.0–7.4 (br s, 3H), 7.5 (br s, 1H), MS (nominal): 303 (M^+), MS (accurate): M^+ calcd: 303.1695, M^+ found: 303.1695.

1-Phenylacetyl-4-(3-guanidopropionyl)piperazine (11). Melting point: 184–185 °C, R_f 0.41 (ButOH:H_2O:AcOH, 4:1:1), ^{1}H NMR (DMSO-d_6): δ 2.6 (t, 2H), 3.3–3.5 (m, 8H), 3.4 (m, 2H), 3.7 (s, 2H), 7.2–7.3 (m, 5H), 6.8–7.4 (br s, 3H), 7.5 (br s, 1H), MS (nominal): 316 (M^+-1), 258 [$(M^+-1)-H_2NCNHNH^{\bullet}$], MS (accurate): M^+ calcd: 317.1852, M^+ found: 317.1858.

1-Phenylacetyl-4-(4-guanidobutyryl)piperazine (12). Melting point: 169–170 °C, R_f 0.43 (ButOH:H_2O:AcOH, 4:1:1), ^{1}H NMR (DMSO-d_6): δ 1.65 (t, 2H), 2.3 (m, 2H), 3.0–3.5 (m, 8H), 3.4 (m, 2H), 3.7 (s, 2H), 7.2–7.3 (m, 5H), 6.8–7.4 (br s, 3H), 7.7 (br s, 1H), MS (nominal): 331 (M^+), 315 ($M^+-H_2N^{\bullet}$), MS (accurate): M^+ calcd: 331.2008, M^+ found: 331.2016.

1-Phenylacetyl-4-(6-guanidohexanoyl)piperazine (13). Melting point: 112–114 °C, R_f 0.47 (ButOH:H_2O:AcOH, 4:1:1), ^{1}H NMR (DMSO-d_6): δ 1.3 (t, 2H), 1.5 (m, 4H), 2.3 (m, 2H), 3.0–3.5 (m, 8H), 3.4 (m, 2H), 3.7 (s, 2H), 7.2–7.3 (m, 5H), 6.8–7.5 (br s, 3H), 7.8 (br.s, 1H), MS (nominal): 359 (M^+), 343 ($M^+-H_2N^{\bullet}$), MS (accurate): M^+ calcd: 359.2321, M^+ found: 359.2316.

Rat aorta relaxation assay

Compounds were dissolved in 25 mM Hepes buffer, pH 7.4 and were diluted appropriately for evaluation in the rat aorta relaxation assay as described in detail elsewhere.[12,13] The activity of the compounds was manifested as a relaxation response in an aorta ring preparation that had been precontracted by the addition of 1 μM phenylephrine (PE). Aorta preparations were used either with an intact endothelium, responsible for the release of nitric oxide; or with the endothelium denuded, in which case, no relaxant activity due to nitric oxide release could be observed. The relaxant activity of all compounds was monitored relative to that of 1 μM acetylcholine (Ach), which requires the presence of an intact endothelium for its nitric oxide-dependent relaxant activity. The relaxant activity of compound **13** was also compared with that of the thrombin receptor activating peptide, SFLLR-NH_2 (P5-NH_2); the nitric oxide-dependent relaxant activity of this peptide is also dependent upon the presence of an intact endothelium. To construct concentration-effect curves for all test compounds, the relaxant activity of each compound was expressed as a percentage (%Ach) of the relaxation caused in the same preparation by 1 μM acetylcholine. Values shown in the concentration-response curves represent the averages (± SEM) of three or more independently measured responses in different tissue preparations. Those compounds lacking activity were evaluated in at least two separate experiments using tissues from different animals.

Platelet aggregation assay

A. Preparation of platelet-rich plasma

Blood was drawn between 8 and 11 a.m. from healthy volunteers, who had not taken medications known to affect platelet function for the past two weeks. Blood venus (30 mL) was collected from a forearm vein, without venous stasis, using a 19-gauge butterfly needle, into a 3.2% trisodium citrate buffer, as anticoagulant (final concentration 10 mM). Blood samples were then centrifuged at 800 rpm at room temperature for 8 min and the supernatant platelet-rich plasma (PRP) was counted by a Coulter counter (Coulter Corp.) and diluted with platelet-poor plasma (PPP) to the desired final concentration (2×10^8 platelets/mL).

B. Platelet aggregation study

Platelet aggregation was determined by the optical method in a four-channel platelet aggregometer (Bio/Data, USA) equipped with an aggregation module PAP-4C. Samples of platelet suspensions (450 μL) under stirring at 1100 rpm were activated with 50 μL of either increasing doses of bovine thrombin (0.1, 0.2, and 0.3 NIU/mL) or 1 μM of SFLLR-NH_2 at 37 °C. Thrombin was obtained from Chromogenix, Sweden and SFLLR-NH_2 was synthesized in our Patras Laboratory.

Light transmittance was measured and recorded for 4 min at which time platelets from most platelet donors had reached a maximal response to thrombin. The results were expressed as the percent change in light transmittance, with light transmittance of plasma alone (PPP) taken as 100% and that of plasma with platelets in suspension taken as 0%. In order to evaluate the biological effects of the non-peptide thrombin receptor mimetics under investigation on platelet aggregation, platelet suspensions were incubated under stirring with the desired concentrations of each compound for at least 4 min prior to the addition of thrombin (0.1–0.3 NIU/mL) or SFLLR-NH_2 (1 μM). Each study was completed within 1 h after blood collection.

C. Calculation of IC_{50} values

The IC_{50} for the data regarding the response to the three levels of compounds (**10**, **11**, and **13**) in different thrombin concentrations (0.2 NIU/mL and 0.25–0.3 NIU/mL) was evaluated using the Random Effects Model (Repeated Maximum Likelihood, or REML method).[32] The independent variable was the percent reductions in optical density (which itself was expressed as a percent) from the baseline value, for various concentrations of the drug (from 0 to 2.0 mM). The dependent variable was the drug concentration. Each patient was considered as a separate variable nested within the response variable and the analysis was performed nine separate times, once for each type-thrombin combination. The individual p-values of each estimate indicate that all are statistically bigger than zero ($p < 0.001$).

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