Potent Inhibitors of Platelet Aggregation Based upon the Arg-Gly-Asp-Phe Sequence of Fibrinogen. A Proposal on the Nature of the Binding Interaction between the Asp-Carboxylate of RGDX Mimetics and the Platelet GP IIb-IIIa Receptor

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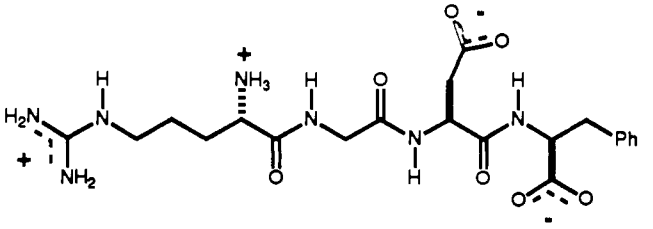
The tripeptide sequence Arg-Gly-Asp (RGD) found in the α chain of fibrinogen (RGDF 95–98, RGDS 572–575) is a key recognition domain for the platelet membrane protein, glycoprotein IIb-IIIa (GP IIb-IIIa; see Figure 1).[1,2] It is now well documented that tetrapeptides containing the RGD sequence are capable of effectively inhibiting the binding of fibrinogen to the GP IIb-IIIa receptor.[3-5] The competitive inhibition of fibrinogen binding prevents platelet aggregation and subsequent white thrombus formation which can lead to arterial occlusion. The RGDX antiplatelet agents show promise for improving the immediate treatment of a myocardial infarct (MI) when used in combination therapy with a fibrinolytic agent (e.g. r-tissue type plasminogen activator or streptokinase).[6]

Previously, we had shown that the inherent inhibitory potency of Arg-Gly-Asp-Phe (RGDF, compound 1)[5] for disrupting the fibrinogen–GP IIb-IIIa interaction can be enhanced 15-fold by removing the Arg-NH_2 and the Arg-Gly amide bond to obtain 8-guanidinooctanoyl-Asp-Phe (GOA-Asp-Phe, compound 2).[8] The removal of the amide bond validates one of Farmer's rules on peptide mimetics in which one maximizes conformational flexibility until a lead compound is discovered.[9] Furthermore, the Phe carboxylate of compound 2 is believed to impart considerable binding energy, since replacement of the phenylalanine residue with phenethylamine (compound 3) resulted in a dramatic decrease in inhibitory potency.[8,10] Herein, we describe the preparation and inhibitory potency of Phe-tetrazole derivative 4 and Asp-tetrazole derivative 5, which lead us to a proposed mode of binding of the Asp-carboxylate of this class of RGDX mimetics to the GP IIb-IIIa receptor protein.



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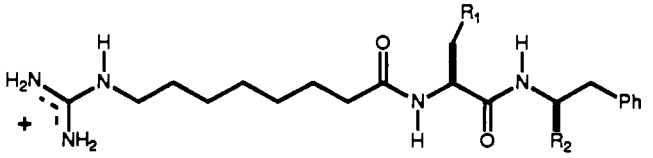

2 $R_1R_2 = CO_2^-$

3 $R_1 = CO_2^-$ $R_2 = H$

4 $R_1 = CO_2^-$ R_2 = Tetrazole

5 R_1 = Tetrazole $R_2 = CO_2^-$

The synthesis of the Phe-tetrazole and Asp-tetrazole derivatives is straightforward as outlined in Scheme I. The Phe-tetrazole mimetic was prepared from the phthaloylphenylalanine amide via dehydration to the nitrile followed by tetrazole formation as previously described.[11] The Asp-tetrazole derivative was prepared by treating the β-cyanoalanine derivative 10 with sodium azide and ammonium chloride. Deprotection of the *tert*-butyl ester required the presence of a *tert*-butyl cation trap, anisole,[12] to prevent the formation of *tert*-butyl tetrazole side products. The guanidine moiety was introduced by treating the free amine in water/dioxane (1:1) with 3,5-dimethylpyrazole-1-carboximidine (1.7 equiv) and Hunig's base followed by purification on reverse-phase HPLC.[13]

Compounds 4 and 5 were tested in competitive binding experiments using ^{125}I-fibrinogen as described by Plow et al.,[3] or alternatively, the inhibition of ADP-induced platelet aggregation was measured in platelet rich plasma (PRP),[5]

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(13) The product was purified on a Waters reverse-phase C-18 μ-Bondapak column (1.9 cm × 15 cm) using a linear gradient of 10% methanol/water, 0.5% acetic acid to 100% methanol (40 min) with a flow rate of 3 mL/min to afford the title compound.

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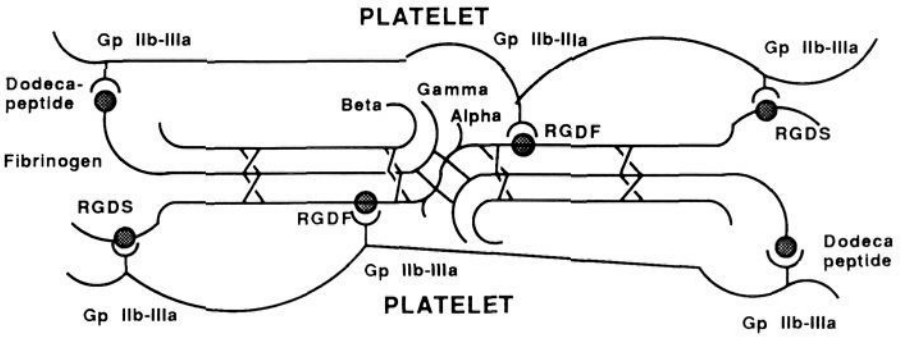
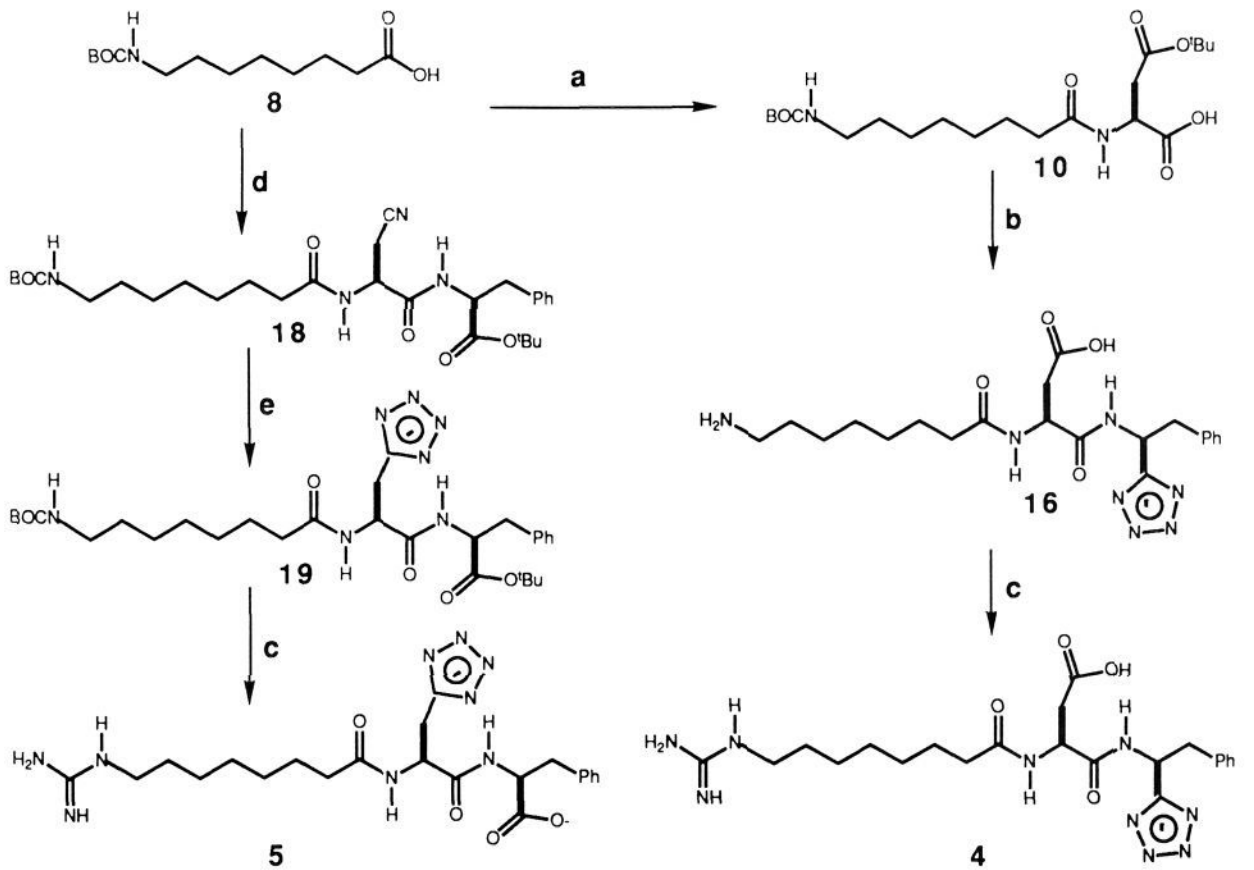


See Ref 7 for similar adaptation.

Figure 1. *Platelet–platelet interaction mediated by fibrinogen.*

Scheme I[a]

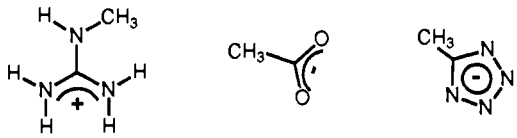


[a] (a) DCC, pentachlorophenol; L-Asp-β-*tert*-butyl ester, DMF; (b) DSC, Phe-tetrazole **7**; (c) 6 N HCl in dioxane/glacial acetic acid (1:1), anisole; guanylation (see text); (d) DCC, pentachlorophenol; β-cyanoalanine, DMF; DCC, L-Phe-*tert*-butyl ester; (e) NaN_3 (10 equiv), NH_4Cl (10 equiv), DMF, 120 °C.

Table I. In Vitro Biologial Evaluation of RGDX Mimics

RGDX mimic	fibrinogen *binding assay:* IC_{50} = [μM]	inhibn of platelet *aggregation (PRP):* IC_{50} = [μM]
1 (RGDF)	9	40
2 (GOA-D-F)	0.6	1.6
3 (phenethyl)	30	60.0
4 (tetrazole-F)	2.0	4.0
5 (tetrazole-D)	inactive	inactive

and the results are summarized in Table I. The Phe-tetrazole derivative **4** resulted in comparable inhibitory potency to compound **2**. The nature of the Phe-carboxylate receptor interaction may possess an electrostatic component and a dipole–dipole component which is supported by comparable activity upon replacement with tetrazole, and slightly less favorable inhibitory potency upon replacement of the carboxylate with carboxamide (6.5-fold less active).[8] However, when the Asp-carboxylate was replaced with tetrazole, the change resulted in a complete abolishment of inhibitory potency (compound **5**). In order to explain these results, one requires a compatible mode of binding of the RGDX mimics to the platelet receptor. In a very elegant study, D'Souza and co-workers found that the region containing the second calcium binding site of GP IIb, residues 296–306 (TD-VNGDGRHDL), is implicated in the ligand binding



11 12 13

Figure 2.

function of GP IIb-IIIa.[14] Furthermore, we were intrigued by their statement that the DGR sequence in this region of the α subunit of integrins is unique to GPIIb. A hypothesis which could explain the lack of activity of the Asp-tetrazole derivative **5** invokes a reinforced ionic interaction (Figure 3) between the carboxylate of **2** and the guanidine of Arg 303 of the receptor which is less favorable with the tetrazole derivative **5**.[9,15,16] The proposed reinforced ionic interaction between the Asp-carboxylate of RGDX mimics and GP IIb imposes considerable geometric constraints on the interacting groups and is supported by the similar loss of activity upon replacement of Asp with glutamic acid or asparagine.[17]

The validity of the above hypothesis was determined through computational studies on model systems which mimic the guanidine–carboxylate and guanidine–tetrazole interactions. The molecules chosen for this study (**11–13**) are illustrated in Figure 2. Models **11–13** were built using MacroModel v3.1 on the personal iris and minimized using the MM2 force field (**11** and **12**) and semiempirical method AM1 (**13**). Interactions between **11** and **12** were modeled in two modes with respect to the guanidine GC-1A (type A), GC-1B (type B; see Figure 3).

The interactions between **11** and **13** were modeled in type A and B modes with respect to the guanidine and two orientations with respect to the tetrazole moiety (C–C bond distal-GT-2, proximal-GT-3) generating the four complexes shown in Figure 4.

The guanidine–carboxylate complexes were initially optimized using the MacroModel MM2 program, while the corresponding complexes with 5-methyltetrazole were optimized using AM1. All the complexes had planar configuration upon initial optimization as would be consistent with their charged states at the physiologic pH. The AM1 and MM2 refined structures of **11–13** and their complexes were then subjected to ab initio calculations using Gaussian 90 with a 6-311G*/RHF basis set. During the ab initio calculations the geometries were kept fixed. The interaction energies were calculated using the simple approximation that the total energy of the complex is equal to the sum of the total energies of the individuals and their interactions (eq 1–3). The energies of the complexes and the individual moieties are listed in Table II. Sub-

Table II. Interaction Energies of the Guanidine–Carboxylate and Guanidine–Tetrazole Complexes

complexes		
11·12	11·13	$E_{11\cdot12} - E_{11\cdot13}$, kcal/mol
GC-1A	GT-2A	−8.31
GC-1A	GT-3A	−3.96
GC-1B	GT-2B	−18.85
GC-1B	GT-3B	−17.53

$$E_{11} + E_{12} + E_{11\cdot12} = E_{C1} \quad (1)$$

$$E_{11} + E_{13} + E_{11\cdot13} = E_{C2} \quad (2)$$

tracting eq 2 from eq 1 gives

$$E_{11\cdot12} - E_{11\cdot13} = E_{C1} - E_{C2} - E_{12} + E_{13} \quad (3)$$

If $(E_{11\cdot12} - E_{11\cdot13})$ is negative, then the interactions between the guanidine and acetate are favored to those between the tetrazole and guanidine.

The results of our computational studies indicate (Table II) that the carboxylate–guanidine interactions are favored over the tetrazole–guanidine interactions by 4.0–18.9 kcal/mol. This could be attributed principally to the differences in charge distribution between the carboxylate and the tetrazole. In **12**, the two oxygens carry a charge of around −0.9 (as determined based on quantum chemically evaluated electrostatic potential with 6-31G* basis set), while the corresponding nitrogens in **13** carry a charge of around −0.2 (Figure 5). These charges are in the isolated systems and will be somewhat reduced in the complexes due to possible "charge transfer effects". The majority of the negative charge in tetrazole resides on the two nitrogens adjacent to the ring carbon (−0.7 on each nitrogen). In light of this delocalization of charges around the tetrazole in **13**, it is easy to rationalize the differences in the energies of interactions in two different orientations of the tetrazole. In an arrangement where the C–C bond in **13** is proximal to the guanidine, the two interacting nitrogens resemble the carboxylate oxygens more closely than do the corresponding nitrogens when the C–C bond is distal.

It must be pointed out that although these calculations have been done in vacuo without the explicit solvent environment, the calculated interaction energies are in qualitative agreement with the lowered affinity of **5** to the GPIIb/IIIa receptor complex. Recent computational studies have demonstrated the importance of including solvation and desolvation energies in studying the relative binding free energies of two molecules with a common receptor;[19] therefore, we have initiated molecular dynamics and free energy perturbation studies with an explicit inclusion of solvent environment to gain a more accurate determination of the contribution imparted by the guanidine–carboxylate interaction to binding strength relative to the guanidine–tetrazole interaction. However, the more detailed computational studies are beyond the scope of this communication, and therefore, the results will be reported elsewhere.

In summary, tetrazole was found to effectively mimic the Phe-carboxylate, but not the Asp-carboxylate of our

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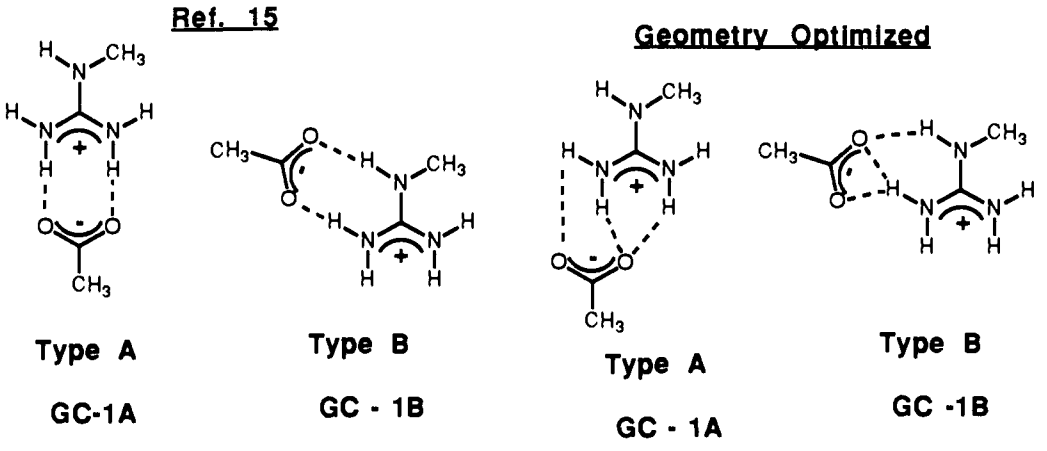

Figure 3.

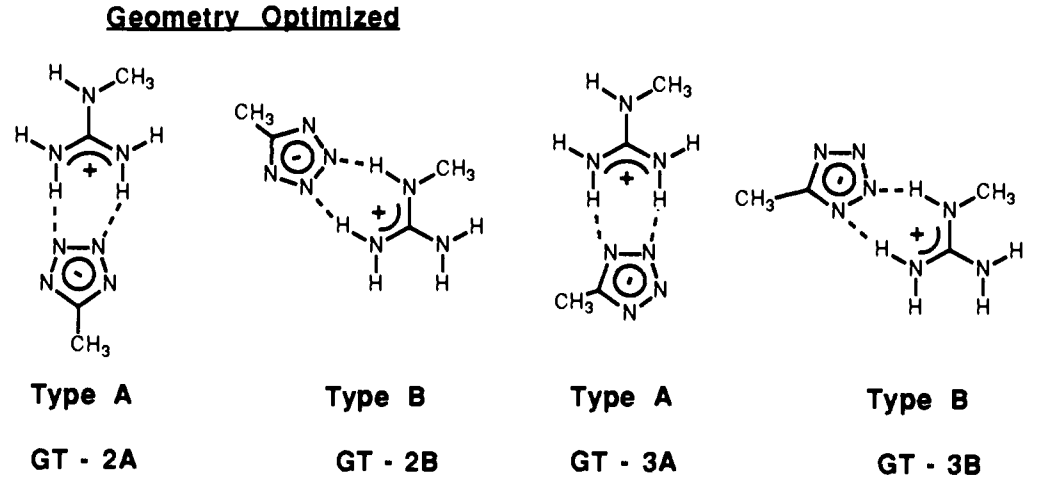

Figure 4.

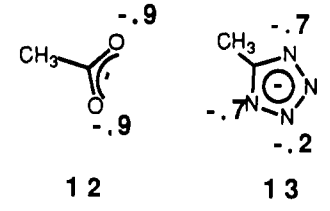

Figure 5.

RGDX mimetic 2. A hypothesis to explain this result invokes a re-inforced ionic complex between the Asp-carboxylate and the guanidine of Arg-303 of GP-IIb. Ab initio calculations on guanidine–carboxylate (GC) and guanidine–tetrazole (GT) complexes are consistent with our hypothesis. The results of this investigation have been used as a platform for model building of complexes between potent antithrombotic agents and a sequence of GP IIb implicated in binding them.[14]

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Supplementary Material Available: Physical (proton and carbon NMR) data for intermediates and final targets **4** and **5** and a detailed description of the pharmacological assays (^{125}I–fibrinogen competitive binding assays and ADP-induced PRP assay) (9 pages). Ordering information is given on any current masthead page.