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REVIEW ARTICLE

Antithrombotic Agents: From RGD to Peptide Mimetics

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Abstract—This review covers the recent advances in the development of highly potent inhibitors of platelet aggregation as potential therapeutic drugs for thrombosis related to cardiovascular and cerebrovascular diseases. The discovery of RGD sequence-directed cell surface receptors (the integrins) has led to extensive research in the development of small RGD containing peptides and their mimetics as antithrombotic agents. These agents work by inhibiting platelet aggregation through competitive blocking of fibrinogen to the platelet surface receptor, GPIIb/IIIa. The pharmacophoric nature of the aspartic acid and arginine side chains of the RGD unit has allowed the development of strategies for rational design, largely based on assumed bioactive RGD conformations and lead optimization. Applications of such strategies, from RGD peptides to peptide hybrids and then to non-peptide mimetics, are described. Also discussed is the important issue of specificity toward GPIIb/IIIa, keeping in view that the RGD unit is a key recognition signal for a variety of cell surface receptors.

1. Introduction

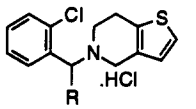
Major advances in antithrombotic research have been made over the past 10 years. These advances follow seminal findings regarding the critical physiological role of blood platelets in relation to vaso-occlusive thrombotic events. As major components of a complex wound-healing system, platelets function by adhering to blood vessel walls at the site of injury and aggregating with each other, forming a hemostatic plug. However, virtually the same processes operating under pathophysiological conditions, can be the cause of cardiovascular, cerebrovascular and peripheral vascular diseases. The root of the matter is the inability of platelets to distinguish a damaged blood vessel wall in need of repair from a thrombogenic surface, such as a fractured atherosclerotic plaque. When such thrombogenic surfaces appear in blood vessels, they elicit the adhesion of platelets on the plaque. This event induces the self-aggregation of platelets, and may eventually lead to vessel occlusion and the interruption of blood flow.[1] Cleavage of the thromboembolic obstruction with thrombolytic agents, such as tissue plasminogen activator or streptokinase, is a common approach for the therapy of acute myocardial infarction. However, the administration of thrombolytic agents is often accompanied by rapid reocclusion linked to the activation of platelets through a number of possible mechanisms, and there is also a high potential for the development of hemorrhagic conditions.[2] Anticoagulant therapy, based on antagonism of vitamin K, necessary for the synthesis of various clotting factors, is

useful in the treatment of conditions such as deep venous thrombosis and pulmonary embolism.[3] However, anticoagulants do not work in acute conditions and require extended periods of closely monitored therapy mainly because of the high risk of bleeding.[3–5] It is clear that neither thrombolysis nor anticoagulation are complete as therapeutic strategies, i.e., drugs with the ability to restrict the development or reoccurrence of thrombosis are equally important. The inhibition of platelet function was viewed as the crucial target in this endeavor. In this general effort, the processes of platelet adhesion and aggregation were identified as appropriate targets by current antithrombotic drug discovery programs. The discovery of the biomolecular processes that govern the phenomenon of platelet aggregation not only lent impetus, but also was central to the unfolding of a completely new generation of antithrombotic drug candidates.[6,7]

1.1. *Antiplatelet therapy and the RGD motif*

Antiplatelet therapy, in general, encompasses all antithrombotic strategies that are based on the inhibition of platelet adhesion and aggregation. One approach to antiplatelet therapy is to block the primary stimulus by any one of several different agonists, e.g., thrombin, epinephrine, ADP, collagen, etc. (Fig. 1). Thrombin, for example, plays a dual role as a platelet activator and catalyst for the conversion of fibrinogen to fibrin in the coagulation cascade. Hirudin from the leech, *Hirudo medicinalis*, is a highly specific and direct inhibitor of thrombin.[8,9] However, hirudin and its analogs exhibit a strong anticoagulant effect, making them difficult to administer in the absence of appropriate antidotes. Ticlopidine (**1a**)[1,10,11] and clopidogrel (**1b**)[10,12] are inhibitors of ADP-induced aggregation, possibly through the blocking of the ADP receptor. Another approach to antiplatelet therapy involves the interruption of the signal transduction mechanism which follows agonist binding to the platelet surface or other perturbation and culminates in platelet activation. One of the well-defined activation pathways is based on the metabolism of arachidonic acid to thromboxane A_2 (**2**) which causes the release of platelet agonists from specific platelet granules besides being an agonist itself.[1,2] Aspirin (**3**) inhibits the first step in the biosynthesis of thromboxane A_2 (**2**) from arachidonic acid in platelets through acetylation of the crucial enzyme, cyclooxygenase.[1] Specific inhibitors of the enzyme, thromboxane synthase, and the thromboxane A_2 receptor have also been developed. However, the specific inhibition of a particular agonist leaves open several alternative routes to platelet activation. Blocking of the arachidonic acid pathway, on the other hand, is not a complete solution, either because (i) all agonists are capable of activating the platelet for aggregation independent of the pathway and (ii) the metabolism of arachidonic acid is not a platelet-specific process. Increase in the level of platelet cyclic-AMP can potentially inhibit all the activation mechanisms. Prostacyclin (**4**), a short-lived endothelium derived biomolecule, directly affects the levels of cyclic-AMP and nitric oxide, allowing control via a non-agonist-dependent route.[13] However, at concentrations required for drug action, prostacyclin can affect several biological processes that depend on cyclic-AMP leading to severe hemodynamic consequences.[14] An ideal antithrombotic agent must, therefore, inhibit platelet aggregation reversibly and specifically regardless of the nature of the agonist and, at the same time, not affect significantly other normal physiological processes.

Adhesion and aggregation of platelets are processes mediated by an array of platelet surface receptors. The interaction of these receptors with adhesive glycoproteins present in subendothelium and in plasma allow attachment of platelets to damaged vessel surfaces or other platelets. Glycoprotein receptors responsible for adhesion include GPIa/IIa (for collagen), GPIc/IIa (for laminin), GPIc*/IIa (for fibronectin), GPIb (for von Willebrand factor) and GPIIb/IIIa. The GPIIb/IIIa receptor has the ability to bind to several glycoproteins and is primarily responsible for platelet aggregation. While GPIIb/IIIa is capable of binding to several glycoproteins, this receptor mainly binds to the multivalent plasma protein, fibrinogen, to cause aggregation.[15–18] Unlike the other adhesive protein receptors, GPIIb/IIIa does not expose its binding sites under normal physiological conditions, i.e., the activation of GPIIb/IIIa is the last step in the chain of events that leads to platelet activation and subsequent aggregation (*vide supra*). Once GPIIb/IIIa is activated, the extremely high density of the receptor on platelets (~50,000 per platelet) ensures rapid aggregation. The inhibition of platelet aggregation by selectively blocking the association of fibrinogen-GPIIb/IIIa has formed the basis of an attractive antithrombotic strategy independent of the various activation pathways without interfering in the function of other receptors.



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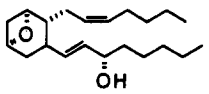

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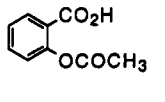

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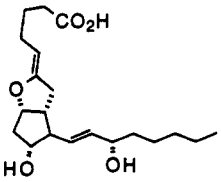

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The GPIIb/IIIa receptor and several other adhesive glycoprotein receptors belong to a superfamily of structurally related glycoprotein receptors present in many different cells, and have been named the 'integrins',[19] or the 'cytoadhesins'.[20] The integrins are non-covalently associated heterodimers of α and β subunits; integrin families are classified based on common β subunits. The collagen, laminin and fibro-nectin receptors belong to the β_1 family, while the GPIIb/IIIa receptor, specific to platelets and megakaryocytes, and the vitronectin receptor, present on platelets and other cell types, belong to the β_3 family.[2] It has been demonstrated that several integrins including GPIIb/IIIa, can recognize a common tripeptide

motif, the Arg-Gly-Asp (RGD) sequence. This sequence is present in a surprisingly large number of adhesive glycoproteins serving diverse functions. The importance of the RGD sequence was confirmed by the inhibition of glycoprotein adhesion in the presence of small RGD-containing peptides.[21] Despite the common recognition sequence, integrins are quite specific in their interaction with different glycoproteins. In their widest scope, the omnipresent integrins and the RGD recognition signal have been implicated as parts of a versatile and subtle cell recognition system controlling crucial events such as cell adhesion, differentiation and even tumor metastasis.[22-24]

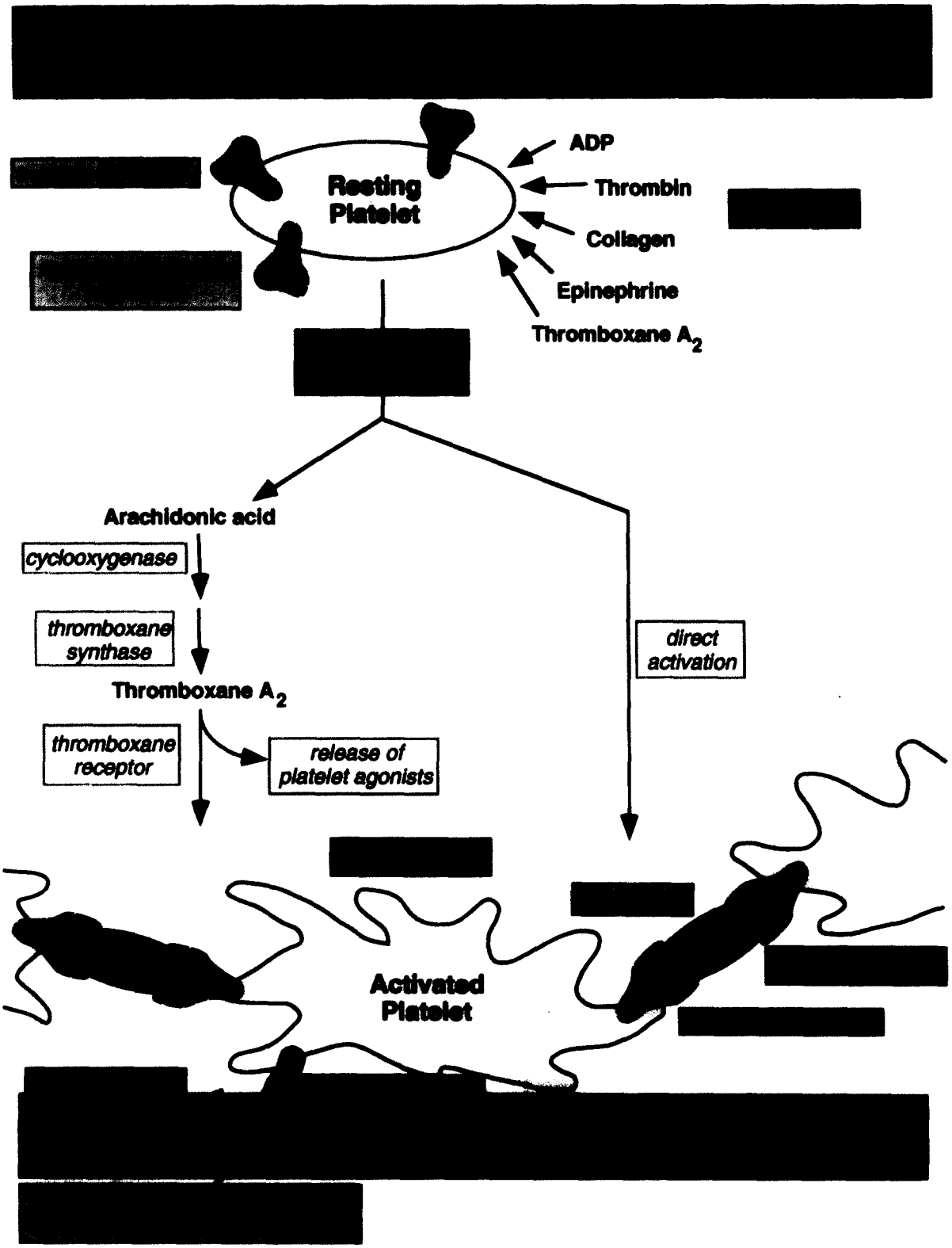


Figure 1. Pictorial representation of the processes of platelet adhesion and aggregation

1.2. *Integrin receptor GPIIb/IIIa*

The GPIIb/IIIa receptor, like other integrins, is a non-covalently associated heterodimeric complex composed of the subunits GPIIb or α_{IIb}(α-subunit) and GPIIIa or β_3 (β-subunit), each coded by a separate gene[25] (Fig. 2). Calcium has been found necessary to maintain the integrity of the heterodimeric structure.[26–28] The N-termini of both subunits protrude into the extracellular matrix providing a zone for interaction with ligand proteins; the C-terminal ends form short cytoplasmic tails that possibly interact with the cytoskeleton.[25] The subunit GPIIIa consists of 762 amino acids.[29] Its extracellular domain contains a cysteine rich, protease-resistant region and a large disulfide loop that links the amino terminus to the mid-region of the extracellular domain.[30] The subunit GPIIb contains 1170 amino acids[31] and 15% carbohydrate,[32] which form a heavy chain linked to a light chain by a disulfide bridge. The heavy chain is completely extracellular. The subunit GPIIIa and the heavy chain of GPIIb associate into a complex that recognizes the RGD sequence.[32]

The GPIIb/IIIa receptor is a two-way signaling molecular complex, a property shared by most integrins.[33] Inside-out signaling from the cell allows the regulation of receptor affinity and conformation. Thus, GPIIb/IIIa undergoes a substantial conformational change on activation to gain a high affinity to fibrinogen, an event essential for platelet aggregation. Ligand binding to integrins, including GPIIb/IIIa, has been demonstrated to be accompanied by extensive tyrosine phosphorylation events within the cell.[33] Unlike other integrins, GPIIb/IIIa is not very specific to fibrinogen,[34] and recognizes other glycoproteins such as fibronectin,[18,35] von Willebrand factor,[36] and vitronectin.[37] However, fibrinogen is the major protein that can bind to GPIIb/IIIa under physiological concentrations of these glycoproteins.[2] Fibrinogen is a dimer of three polypeptide chains, the α-, β- and γ-chains. This dimeric structure of fibrinogen allows the binding of juxtaposed platelets to each half of the dimer thus mediating aggregation.[38] Each of the Aα chains of fibrinogen contains two RGD recognition sequences, i.e., RGDF units at Aα95–98 and RGDS units at Aα572–575.[16,17,25] It also contains a GPIIb/IIIa-specific dodecapeptide recognition sequence, HHLGGA-KQAGDV, at the C-terminus of each of its γ-chains.[39,40] It has been suggested in a binding model that all six recognition sites on fibrinogen participate in binding to GPIIb/IIIa.[41] It should be noted that recent findings based on recombinant human fibrinogen mutants have indicated the role of the γ-chain recognition sequence essential for fibrinogen binding to GPIIb/IIIa.[38,42] Competitive binding to GPIIb/IIIa has been observed between RGD peptides and γ chain peptides, suggesting that (i) these two peptide types compete for the same binding site or (ii) there is some functional interaction between two different binding sites for each peptide type.[43,44] The exact nature of the GPIIb/IIIa binding sites for fibrinogen remain to be clarified. From the standpoint of drug design, however, the RGD sequence has provided a solid basis for the successful development of highly potent antagonists for the fibrinogen binding to GPIIb/IIIa.

1.3. *Inhibitors of GPIIb/IIIa–fibrinogen association*

The possibility of inhibiting platelet aggregation based on the GPIIb/IIIa receptor antagonism was first explored

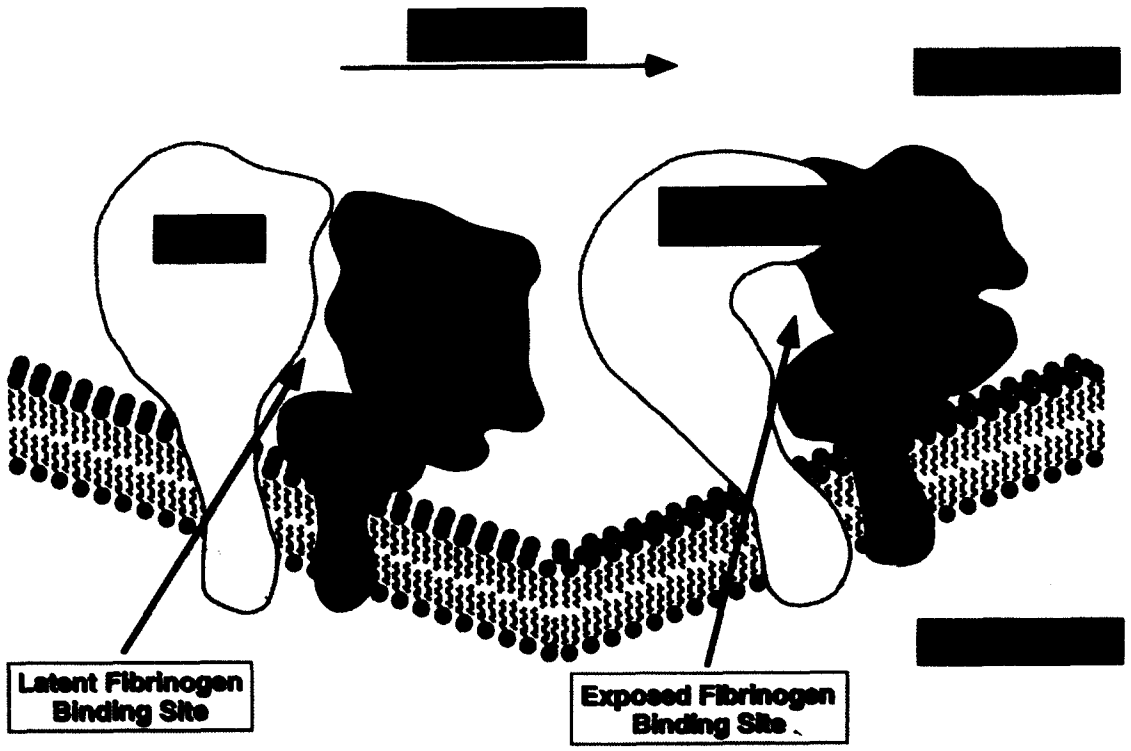


Figure 2. Diagrammatic representation of platelet receptor GPIIb/IIIa.

using murine monoclonal antibodies, at the time when very little was known about the nature of the fibrinogen–GPIIb/IIIa interaction.[45,46] Subsequent research on monoclonal antibodies has provided deep insight into the function of GPIIb/IIIa as related to platelet aggregation besides providing clinically successful antithrombotic agents. The monoclonal antibody 7E3 (Centocor, Malverne, PA) underwent evolution through fragmentation to $F(ab')_2$, and then to Fab fragments showing lower antigenic response.[47] A chimeric version of the murine-derived 7E3 Fab fragment, c7E3 Fab, was developed, that involved the humanization of the murine constant domains. The c7E3 Fab fragment elicited a remarkably low immunogenic response compared to m7E3 Fab and is now registered for clinical use.[48,49] The 7E3-based monoclonal antibodies have been clinically evaluated for the treatment of unstable angina, acute myocardial infarction and the ischemic complications of coronary angioplasty.[50] Following two pilot studies on c7E3 Fab,[51,52] a large scale Phase III clinical trial was conducted by the EPIC (Evaluation of 7E3 in the Prevention of Ischemic Complications) investigators on 2,099 high-risk patients receiving coronary angioplasty.[49] The efficacy of c7E3 Fab was demonstrated in reducing ischemic complications following angioplasty based on a reduction of clinical end points by 35%. However, this was accompanied by a significant increase in bleeding events which required transfusions. An assessment between the benefit of treatment depending on the severity of the condition and the risks of bleeding is necessary before clinical administration. There have also been reports of increases in bleeding time in human and animal trials[53,54] although it is now quite clear that measurement of bleeding time does not directly correlate with incidences of hemorrhagic events.[55,56] The other disadvantages normally associated with the administration of monoclonal antibodies, including immunogenicity and thrombocytopenia, appear to have been greatly reduced, particularly with the administration of c7E3 Fab. There are also no reports of adverse cross-reactivity with other receptors. However, the continuation of careful monitoring of these potential hazards in future studies is required.

A family of homologous peptides obtained from snake venoms, named the disintegrins, has been found to possess RGD units, and its members are among the most potent inhibitors of GPIIb/IIIa known. The disintegrins, named after their ability to inhibit integrin function, usually in a nonselective manner, have been studied in great detail (*vide infra*). These studies have provided information about the conformation of the RGD moiety that could be used for the development of conformationally restricted inhibitors of platelet aggregation.[57–59] Based on the hypothesis that the RGD sequence plays a key-role, i.e., as a pharmacophore, in the binding of fibrinogen to GPIIb/IIIa, various small RGD-containing peptides have been synthesized which exhibited remarkable potency as the GPIIb/IIIa receptor antagonists. However, the problems of bioavailability, biolability and immunogenicity that are characteristic of peptide-based drugs have spurred research in the direction of the development of smaller and progressively non-peptidic compounds as orally active drugs. For the purpose of blood storage, however, effective agents with shorter half-lives are required and peptides are better suited for this purpose.

This review covers the development of RGD peptides as efficient inhibitors of GPIIb/IIIa–fibrinogen complex formation, as well as conceptually different strategies that have been applied for the rational design of RGD peptide hybrids, peptide mimetics and non-peptide mimetics (see also recent reviews by Blackburn and Gadek,[60] Ojima *et al.*,[61] Cook *et al.*[62] and Weller *et al.*[63]).

2. Disintegrins: RGD-Containing Peptides Isolated from Snake Venom and Leech Proteins

Most of the snake venom proteins obtained from the Viperidae family are homologous and are generally rich in cysteine, implying several disulfide bridges and a cross-linked rigid structure. Several papers have appeared describing the three-dimensional solution structure of echistatin,[64–68] obtained from *Echis carinatus*.[69,70] This protein consists of a well-defined, though irregular, core consisting of several non-classical turns stabilized by hydrogen bonds and disulfide bridges.[68] The RGD unit, however, is present at the tip of a hairpin loop in a flexible and highly exposed part of the molecule, whose stem has recently been characterized and found to be made of two rigid, hydrogen-bonded strands.[68] The RGD unit of kistrin, from the Malayan pit viper *Agkistrodon rhodostoma*, is located similarly at the apex of a long flexible loop.[71] Although not substantiated, an indirect interpretation of NMR data suggested that the RGD-containing loop exists in a relatively extended conformation.[72]

Stronger evidence for the bioactive conformation of the RGD sequence has come forward in the structural analysis of decorsin, a disintegrin obtained from the leech *Macrobdella decora*.[73] The RGD unit in decorsin, unlike the other disintegrins, was found to be very well-defined, perhaps owing to the presence of two proline residues closely flanking the RGD unit, with the Arg and Asp side chains pointing almost in opposite directions. The Gly and the Asp residues appear to be in the $i+1$ and $i+2$ positions of a distorted type II' β-turn.[73] However, this conformation does not impart selectivity, insofar as decorsin, like the other disintegrins, strongly binds to GPIIb/IIIa as well as the vitronectin receptor.[73]

A comparative homology study of snake venom–derived disintegrins with equipotent leech–derived proteins, decorsin and the ornatins, has disclosed a distinct lack of similarity except the amino acid sequence at the apex of the RGD-containing putative binding loop[74,75] (Scheme 1). This fact strongly substantiates the hypothesis of the RGD unit being, in itself, the determinant of binding affinity without controverting any underplaying secondary or alternative binding site hypothesis. This confirms the usefulness of a pharmacophore concept primarily based on the RGD sequence.

Ornatin A3	IPQ**C**ADIKESGQPNDK **C**R**C**NGITCTVGKCKIG**RGD**DNDK **C**T
Ornatin B	IYVRPTNDELNY **C**GDFRELGQPDKK **C**R**C**DGKPCTVGRCKFA**RGD**NDDK **C**ISA
Decorsin	APRLPQ**C**Q-----GDDQEK **C**L**C**NKDECPPGQCRFP**RGD**ADPY **C**E
Kistrin	GKECDCSSPENPCCDAATCKLRPGAQCGEGLC**C**EQCKFSRAGKICRIP**RGD**MPDDRCTGQADCPRYH
Echisatin	ECESGPC**C**RNCKFLKEGTICKRA**RGD**DMDDYCNGKTCDCPRNPHKGPAT

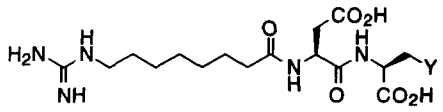
Scheme 1. Amino acid sequence homology for snake venom and leech derived disintegrins.

Although the development of RGD-based GPIIb/IIIa antagonists has been the focus in this field, it may be of interest to note here that a non-RGD-containing peptide inhibitor of platelet aggregation has been isolated from the tick *Ornithodoros moubata*, and named 'disagregin' (IC_{50} = 0.123 μM PRP/ADP).[76] Disagregin has been shown to bind to both GPIIb and GPIIIa subunits, and also to unstimulated as well as ADP-stimulated platelets. This peptide does not show any homology with other known disintegrins.[76]

3. Acyclic Semipeptides and the RGD Pharmacophore Hypothesis

RGD-containing peptides were instrumental in the elucidation of the binding characteristics of fibronectin with cell surfaces. The role of the RGD unit was first noticed after sequencing of the 108-amino acid cell attachment domain of fibronectin was accomplished; the synthesis of progressively smaller peptides containing parts of the same sequence eventually established the RGDS unit as the minimum requirement for activity.[77] It was later revealed that the RGD tripeptide sequence was the absolute minimum recognition sequence for cell attachment activity while the serine residue was replaceable.[77] This minimum requirement was confirmed by the substitution of arginine, glycine, and aspartic acid with other amino acids as well as by reversal of the sequence (DGR).[16] Although the substitution of L-Arg with D-Arg did not show much effect, the replacement of the glycine residue with D-Ala or that of L-Asp with D-Asp, resulted in totally inactive peptides.[78] Similar RGD-containing peptides were used as eluent material in affinity chromatography of cell extracts to obtain RGD-associated platelet receptors, including GPIIb/IIIa.[36] The potential use of RGD-containing peptides as antithrombotic agents was established by the ability of RGD-containing peptides to inhibit platelet aggregation *in vitro*,[17] and prevent thrombus formation *in vivo* in dog by intracoronary administration.[79]

The identification of the RGD sequence as a pharmacophore represented the first stage of the development of a peptide model for rational design, i.e. once the necessity of the RGD sequence was established, attention was focused on the amino acid residues flanking the RGD group. It has been shown that introduction of a hydrophobic residue at the C-terminus of the RGD moiety increases inhibitory activity.[59,80-85] For example, it has been shown that RGDW (IC_{50} = 14 μM, human PRP/ADP) is superior to RGDF (IC_{50} = 37 μM, human PRP/ADP), RGDV (IC_{50} = 150 μM, human PRP/ADP) and RGDS (IC_{50} = 170 μM, human PRP/ADP) which is consistent with the decreasing order of hydrophobicity.[81] This trend has been further confirmed in the structure–activity relationships of RGD-based semipeptide **5** that contains different hydrophobic groups in an analogous position.[86] Notable in the semipeptide **5** is the replacement of the arginine residue with a non-peptidic 8-guanidinooctanoyl group. For the substituent Y in **5**, an indolyl group is found to be more effective than a 1-naphthyl or a phenyl group (**5**: Y = indolyl, IC_{50} = 0.85 μM; Y = 1-naphthyl, IC_{50} = 1.2 μM; Y = phenyl, IC_{50} = 1.6 μM, human PRP/ADP).[86]



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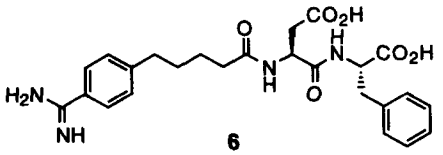
It has been shown that the activity of RGDS is improved upon acetylation of the N-terminus at arginine and amidation of serine at the C-terminus.[87] This peptide, Ac-RGDS-NH_2 (IC_{50} = 91.3 μM, canine PRP/ADP), proved to be a lead in the development of certain cyclic RGD peptides which will be discussed in the following section. It is also evident from previous studies on inhibitors of serine proteases, e.g., trypsin and thrombin, that the *p*-amidinophenyl moiety is a suitable mimic for the arginine side chain.[88-90] Thus, Zablocki *et al.* reported that the guanidino moiety of arginine in RGDS could be mimicked by a *p*-amidinophenyl group to yield highly active analogs,[91] which eventually led to the development of **6** (SC-52012, IC_{50} = 0.042 μM, human PRP/ADP). The semipeptide SC-52012 (**6**) is currently under phase I clinical trials as an intravenous drug to potentiate thrombolysis, prevent acute reocclusion and treat unstable angina.[91]

Alig *et al.* independently reported the use of the *p*-amidinophenyl group for the modification of RGDV,[92] e.g., **7** (IC_{50} = 0.3 μM, human PRP/ADP). Kottirsch *et al.*

also reported a potent analog **8** (IC_{50} = 0.040 μM, human PRP/ADP) that further incorporated a γ-lactam surrogate in order to constrain the Gly–Asp amide bond.[93]



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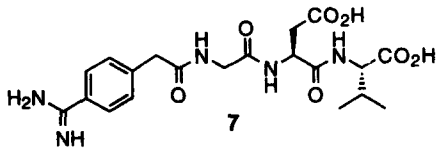

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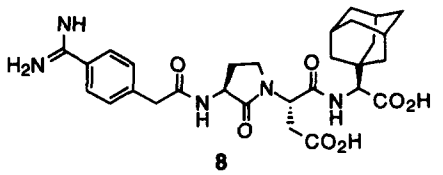

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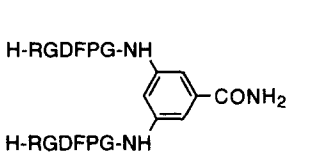
Multi-strand RGD peptides have been studied in these laboratories using a variety of branching linkers to examine the effects of multi-RGD units per molecule on the inhibitory activity against platelet aggregation.[94] In an effort to stabilize the peptides against aminopeptidases, several acyl groups were introduced at the arginine N-terminus of the double-strand RGD peptides.[94] These peptides were found to possess significantly better activity than that of non-acylated peptides, e.g., **9b** (IC_{50} = 0.35 μM, human PRP/ADP) and **10** (IC_{50} = 0.087 μM, human PRP/ADP) in comparison with **9a** (IC_{50} = 13 μM, human PRP/ADP).[94] Based on the rational simplification of **10** and a structure–activity relationship study, RGD-semipeptides **11** and **12** have been developed. These semipeptides contain tryptophan as the strategic hydrophobic binding moiety flanking the Asp residue at the C-terminus.[82] These semipeptides are highly active inhibitors of platelet aggregation, ranking amongst the most potent inhibitors known to date (**11**, IC_{50} = 0.070 μM, human PRP/ADP; **12**, IC_{50} = 0.026 μM, human PRP/ADP).[82] It has also been shown that a semipeptide containing Phe instead of Trp in **12** possesses comparable activity (*vide infra:* **67**, IC_{50} = 0.05 μM, human PRP/ADP).[95]

The development of acyclic semipeptides based on the RGD pharmacophore was the first and extremely crucial step in the rational design of peptide mimetics capable of selective binding to GPIIb/IIIa. The next step was the development of more constrained molecules in an effort to better define the conformational requirements of the RGD pharmacophore, because the high flexibility of small linear peptides usually militates against unambiguous description of their conformational properties. Nevertheless, there are a few examples of conformational studies on small linear RGD peptides. For example, a Monte Carlo simulation employing NMR constraint data indicates a high probability of type II β-turn in GRGDS.[96] A more recent study on the conformations of RGDW and rGDW (r = D-Arg) has revealed the presence of type II' β-turns in both peptides.[97]

At this stage, it was recognized that certain problems characteristic of peptide-based agents, e.g., potential immunogenicity and low bioavailability, should be solved in order to accomplish the ultimate goal of developing orally active antithrombotic drugs. In the pursuit of this objective, two different but interrelated approaches have emerged. The first approach is based on the cyclization of RGD peptides and their study as constrained models for mapping out the RGD pharmacophore. The second approach is the optimization of acyclic RGD peptides and their hybrids into non-peptidic molecules through rational design. The cyclic RGD peptides have served as leads for the development of non-peptide mimetics of the RGD sequence.

4. Cyclic RGD Peptides: Elucidation of the RGD Pharmacophore

Although the NMR studies on the structures of disintegrins appear to be too complicated to provide hypothetical scaffolds for conformationally restrained non-peptidic molecules, these studies have probed the



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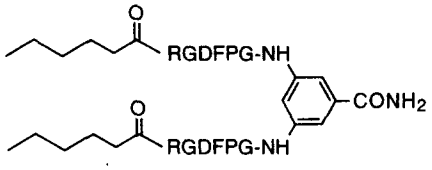

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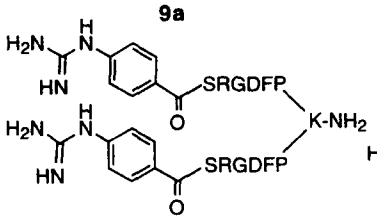

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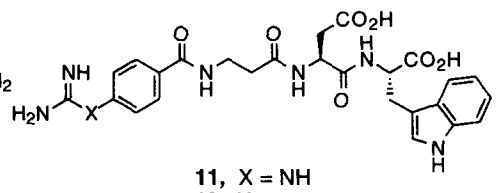


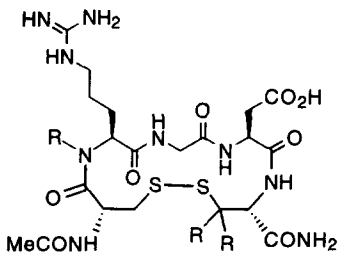
11, X = NH
12, X = none

possibility of describing a single bioactive conformation of the RGD pharmacophore with some success. The cyclization of RGD-containing peptides can greatly reduce the degrees of conformational freedom, and thus provide an excellent opportunity to define the bioactive conformation, if any. In fact, cyclic RGD peptides have shown markedly higher activity than their linear counterparts in several cases. Accordingly, the bulk of conformational studies on the RGD pharmacophore has been performed using cyclic RGD peptides. This section covers the structure–activity relationships and conformational analyses of cyclic RGD peptides and their derivatives.

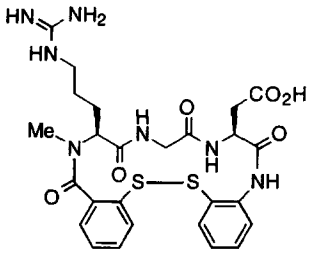
Ali *et al.*, in a detailed investigation on cyclic RGD-containing pentapeptides, have uncovered several structural requirements for the cyclic scaffold which regulate the activity of the peptides.[80] Starting from linear *N*-acetyl-RGD peptide amides such as Ac-RGDS-NH_2 (IC_{50} = 91 μM, canine PRP/ADP; K_i = 4.2 μM, [I^{125}]Fg-GPIIb/IIIa binding), several disulfide linked cyclic RGD peptides were synthesized.[87] To the prototype cyclo-*S,S*-[Ac-CRGDC]-NH_2 (**13**, IC_{50} = 16.2 μM, canine PRP/ADP; K_i = 0.78 μM, [I^{125}]Fg-GPIIb/IIIa binding) were added several constraints in order to limit the mobility associated with the disulfide bond (see Scheme 2) as well as the arginine residue. Thus, N^{α}-methylation of the arginine residue in **13** and replacement of the C-terminal cysteine residue with a penicillamine (Pen) residue bearing *gem*-dimethyl group were carried out to give cyclo-*S,S*-[Ac-C(N^{α}-Me)RGD-Pen]-NH_2 (**14**, SK&F 106760, IC_{50} = 0.37 μM, canine PRP/ADP; K_i = 0.058 μM, [I^{125}]Fg-GPIIb/IIIa binding),[87] which led to a 45-fold increase in platelet aggregation inhibitory activity.

Substitution of the Cys-Pen moiety of **14** with Pen-Pen did not increase the activity any further. However, the replacement of the Cys-Pen moiety by a disulfide-linked (2-mercapto)phenyl residue brought about a further increase in the inhibitory activity as well as affinity for GPIIb/IIIa, viz., this modification eliminates two chiral centers in **14**, increases the lipophilicity, and constrains the χ_1 torsion angles to 0° (see Scheme 2) to yield a highly active cyclic peptide derivative, cyclo-*S,S*-[Mba-(N^{α}-Me)RGD-Man][80,98] (**15**, SK&F 107260, IC_{50} = 0.09 μM, canine PRP/ADP; K_i = 0.0021 μM, [I^{125}]Fg-GPIIb/IIIa binding; Mba = mercaptobenzoyl, Man = mercaptoanilide).

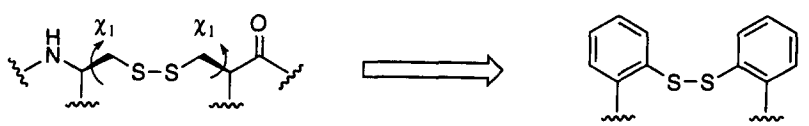
The NMR and distance geometry search-based conformational analysis of **14** and **15** and related compounds yielded interesting results. The backbone conformation appeared to dictate activity insofar as it regulated the relative orientation of the C^{α}–C^{β} vectors of the arginine and aspartic acid residues. The most active analogs were found to exhibit a 'turn-extended-turn' conformation with a C^{α}_{Asp}–C^{α}_{Arg} distance of greater than 6.8 Å.[99] The result appears to be also in consonance with the relatively extended structure predicted for the more flexible snake venom proteins. This 'turn-extended-turn' conformation was further supported by the conformational analyses and X-ray crystallography of these cyclic RGD peptides.[99,100] The crystal structure of SK&F 107260 (**15**) has revealed a highly extended conformation around the Gly residue, with the flanking (N^{α}-Me)-Arg and Asp residues occupying the i+2 and i+1 positions of the β-turns, respectively.[100] NMR study in solution and distance geometry calculations have identified two slowly interchanging conformers of **15**, satisfying NOE distance requirements. Both conformers include the 'turn-extended-turn' conformations having an extended conformation at Gly and a C_7 turn at Asp, similar to the crystal structure.[100] The extended conformation at the Gly residue was also observed for SK&F 106760 (**14**). However, it has been shown that the molecule is not as rigid as **15** and, therefore, other less extended conformations cannot be ruled out.[100] The crystal structure and the major conformer both exhibited 'positive chirality' at the disulfide bond (χ_{SS} ~ +90°).[100] Very similar results were obtained for **15** using a genetic algorithm approach supported by constrained molecular dynamics.[101] This conformational analysis of **15** has been used for developing non-peptidic mimetics of the RGD sequence (*vide infra*).[83,102]



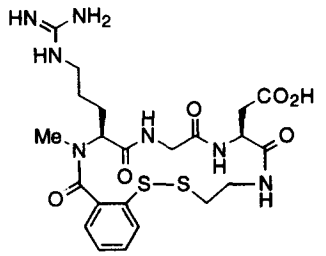
13: R = H
14: R = CH_3 (SK&F 106760)



15 (SK&F 107260)



Scheme 2. Restriction of disulfide χ_1 torsion angles to 0° by incorporation of rotatable bonds in aromatic rings.



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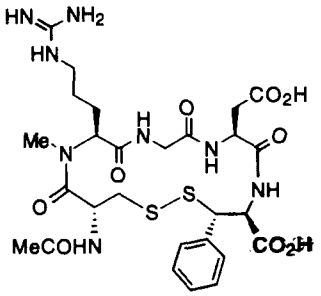
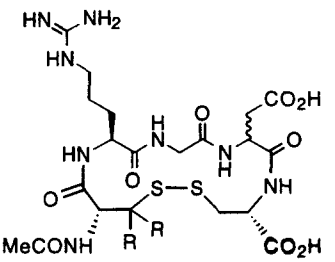


17

It should be noted that single 2-mercaptophenyl substitution at the Cys-Pen moiety does not bring about appreciable improvement in the activity. The mercaptoaniline residue can be considered either as a possible site for receptor interaction or as a major conformational regulator. Replacement of this residue with 2-mercaptoethylamine resulted in significant loss in the activity (**16**, IC_{50} = 0.236 μM, canine PRP/ADP; K_i = 0.0095 μM, [I^{125}]Fg-GPIIb/IIIa binding).[80] It has also been shown that the substitution of the penicillamine residue in **14** with β-phenylcysteine stereoisomers gives the corresponding cyclic peptide derivatives possessing the activity comparable to **15**, e.g., cyclo-*S,S*-[Ac-C(N^{α}-Me)RGD-(2*R*,3*S*)-(β-Ph)C]-OH (**17**, IC_{50} = 0.04 μM, canine PRP/ADP; K_i = 0.0021 μM, [I^{125}]Fg-GPIIb/IIIa binding) which is the most active molecule in the series.[80] This result suggests that the GPIIb/IIIa receptor interacts with the phenyl group of the β-phenylcysteine residue, which is in consonance with several reports invoking the existence of a possible hydrophobic binding site in GPIIb/IIIa in addition to the ionic binding sites for the RGD pharmacophore.[80-82]

Unlike the case for the arginine residue, sequential N-methylation at the Gly and Asp residues resulted in loss of activity. It has been shown that N-methylation of these residues does not cause perturbation of the hypothetical receptor-bound conformation of **15**.[80] The effect, therefore, appears to be due to an unfavorable steric positioning of the methyl group. Replacement of the N^{α}-methyl group of the arginine residue with an N^{α}-ethyl or N^{α}-benzyl group resulted in a modest loss of potency in both derivatives and a significant loss in receptor affinity for the latter.[80] Inversion of the chiral center in the L-Asp residue led to complete loss of the activity and receptor affinity. The replacement of L-(N^{α}-Me)-Arg by D-(N^{α}-Me)-Arg led to the loss of inhibitory activity.[80]

Bogusky and co-workers have reported the conformational analyses of cyclo[Ac-CRGDC]-OH (**18**, IC_{50} = 0.68 μM, human PRP/ADP) and its inactive D-Asp analog (**19**, IC_{50} > 100 μM, human PRP/ADP),[103] which are similar to those reported by Ali *et al.*,[80] based on 2D NMR and molecular modeling. It has been shown that the RGD backbone exists mainly in an extended conformation (C^{α}_{Arg}–C^{α}_{Asp} ≡ 6–7 Å). The major difference between the L-Asp and D-Asp analogs is ascribed to the quasi-dihedral angle of the C^{α} and C^{β} vectors of Arg and Asp with respect to the backbone (~ 0° for the L-isomer and –40° to –100° for the D-isomer), that may well reflect the difference in the activity.[103] The NMR and molecular modeling studies of the more active penicillamine analog of **18**, cyclo[Ac-Pen-RGDC]-OH (**20**, IC_{50} = 0.55 μM, human PRP/ADP), have been carried out in an attempt to define the conformational disposition of the disulfide linkage.[104] However, no conformer is able to fully satisfy the distance constraints based on NOEs because of the mobility of the disulfide linkage as well as the RGD segment. Nevertheless, **20** is found to have a more rigid backbone than **18** on the basis of the fact that **20** possesses a more uniform T_1 relaxation time under variable temperature conditions than **18**. Analogously to **20**, the RGD segment is extended and the C^{α}–C^{β} vectors of Arg and Asp are eclipsed.[104]



18: L-Asp, R = H
19: D-Asp, R = H
20: D-Asp, R = CH_3

Barker *et al.* reported a series of cyclic RGD peptide derivatives bearing thioether, sulfoxide and sulfone linkages as the inhibitors of platelet aggregation.[105] As a modification of Ac-GRGDV-OH (IC_{50} = 77 μM, human PRP/ADP; IC_{50} = 0.012 μM, ELISA assay, GPIIb/IIIa-Fg binding), cyclo-*S*-[Ac-GRGDC]-OH (**21**, IC_{50} = 5.0 μM, human PRP/ADP; IC_{50} = 0.0042 μM, ELISA assay, GPIIb/IIIa-Fg binding) was synthesized, that served as the lead for other cyclic RGD peptides: (for the diastereomer A series, ELISA) **22**, IC_{50} = 0.0014 μM; **23**, IC_{50} = 0.0015 μM; **24**, IC_{50} = 0.004 μM; **25**, IC_{50} = 0.0054 μM; (for the diastereomer B series, ELISA) **22**, IC_{50} = 0.0053 μM; **23**, IC_{50} = 0.015 μM; **24**, IC_{50} = 0.180 μM; **25**, IC_{50} = 0.021 μM.[105] This series of cyclic RGD peptides has revealed that the introduction of a 1-naphthyl (**22**, IC_{50} = 0.0014 μM, ELISA) or a phenyl (**23**, IC_{50} = 0.0015 μM, ELISA) group at the acetyl linker leads to *ca* three-fold enhancement in the activity, and the configuration of the

chiral center exerts marked influence on the affinity to the receptor. Replacement of the Gly residue at the N-terminus with hydrophobic D-amino acids enhances the activity and receptor affinity, whereas the corresponding L-amino acids result in loss of activity.[105]

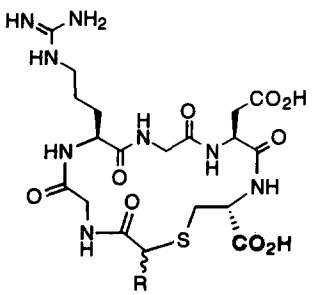
In the corresponding series of cyclic RGD peptides with sulfoxide linkages, cyclo-(*S*)-*S(O)*-[yRGDC]-OH (**26a**, G-4120, IC_{50} = 0.15 μM, human PRP/ADP; IC_{50} = 0.0015 μM, ELISA) (y = D-Tyr) is the most active analog.[105] The (*R*)-*S(O)*-isomer (**26b**) is found to be 15-fold less active. The inhibitory potency, as well as affinity for the GPIIb/IIIa receptor of **26a** (G-4120), compares well with the snake venom kistrin (IC_{50} = 0.15 μM, human PRP/ADP; IC_{50} = 0.002 μM, ELISA). As described above, it has been observed that the carboxylic acid C-terminus gives better activity than the corresponding carboxamide C-terminus for linear RGD peptides and relatively flexible cyclic RGD peptides such as **17**. However, for more conformationally restricted cyclic RGD peptides such as **26a**, the carboxamide terminus is as equally potent as the carboxylic acid terminus.[105]

NMR and molecular modeling studies of G-4120 (**26a**) have revealed a remarkably stable conformation in water.[57] It has also been shown that **26b**, which is 15 times less active than **26a**, adopts a more conformationally averaged structure. The structure of **26a** is defined in part by a type II' β-turn with D-Tyr and Arg occupying the *i*+1and *i*+2 positions, respectively. It appears that this β-turn and the sulfoxide stereogenicity (**26a** has *S* configuration) allows the formation of a hydrogen-bonded network leading to the 'cup-shaped' conformation for the RGD unit.[57] Inversion of the chiral center in D-Tyr of **26a** leads to an inactive molecule, which may be due to the fact that this stereochemical change disrupts the stable type II' β-turn.[57] It is hypothesized that the hydrophobic side chains of D-Tyr and Cys form a shield around the RGD pharmacophore and protect it from solvation, thereby enhancing binding affinity for GPIIb/IIIa.[57] This hypothesis is supported by the fact that the substitution of D-Tyr with hydrophilic amino acids leads to inactive molecules.[105] This cup-shaped conformation is further supported by ensemble molecular dynamics in an effort to derive a consensus conformation of the RGD pharmacophore for the design of peptide mimetics.[106] The proposed 'cup-shaped model' stands out as unique among all proposed conformational models for cyclic RGD peptides most of which emphasize an extended RGD backbone.

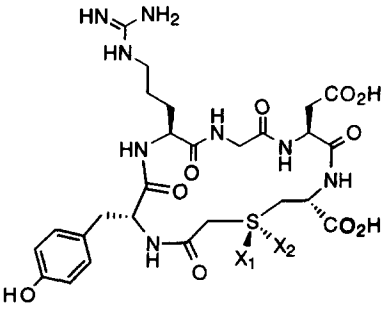
The structure–activity–selectivity relationships of several disulfide-bridged cyclic RGD peptides were investigated, especially those of various amino acid residues flanking the RGD sequence.[107,108] It was found that G-[cyclo-*S,S*-{(Pen)GHRGDLRC}]A-OH (**27**, IC_{50} = 13.7 μM, human PRP/ADP) selectively binds to the GPIIb/IIIa receptor ($\alpha_{IIb}\beta_3$) as compared with its binding to the vitronectin receptor ($\alpha_v\beta_3$[107] or $\alpha_v\beta_5$[108]) and fibronectin receptor ($\alpha_5\beta_1$).[107] Replacement of the penicillamine residue by a more constrained β,β-pentamethylenecysteine (Pmc) led to more potent analogs such as *R*-[cyclo-*S,S*-{(Pmc)GHRGD(Y-OBu^n)RC}]R-OH (**28**, IC_{50} = 0.35 μM, human PRP/ADP), *R*-[cyclo-*S,S*-{(Pmc)GHRGD(Y-OMe)RC}]R-OH (**29**, IC_{50} = 0.56 μM, human PRP/ADP) and *R*-[cyclo-*S,S*-{(Pmc)GHRGD(2-Nal)RC}]R-OH (**30**, IC_{50} = 0.44 μM, human PRP/ADP) [2-Nal = (2-naphthyl)alanine].[108] Removal of the arginine at the N-terminus, replacement of the pentamethylene cysteine with *N*-acetylcysteine, and introduction of proline in place of the histidine residue led to the more active and several-fold more selective carboxamides, cyclo-*S,S*-[Ac-CNPRGD(Y-OMe)RC]-NH_2 (**31**, TP 9201, IC_{50} = 0.22 μM, human PRP/ADP; selectivity, $\alpha_{IIb}\beta_3/\alpha_5\beta_1$ = 283, $\alpha_{IIb}\beta_3/\alpha_v\beta_5$ = 162) and cyclo-*S,S*-[Ac-CNPRGD(Y-OBu^n)RC]-NH_2 (**32**, IC_{50} = 0.10 μM, human PRP/ADP; selectivity, $\alpha_{IIb}\beta_3/\alpha_5\beta_1$ = 250, $\alpha_{IIb}\beta_3/\alpha_v\beta_5$ = 1666).[108]

It should be noted that replacement of the arginine residue flanking the C-terminus of **31** with lysine (**33**, IC_{50} = 1.0 μM, human PRP/ADP), glutamic acid (**34**, IC_{50} = 3.4 μM, human PRP/ADP), and leucine (**35**, IC_{50} = 7.6 μM, human PRP/ADP) results in a substantial decrease in the activity.[108] This arginine residue seems to be playing a key role in the control of template bleeding as well,[109] which implies the existence of another ionic binding site in the GPIIb/IIIa receptor.

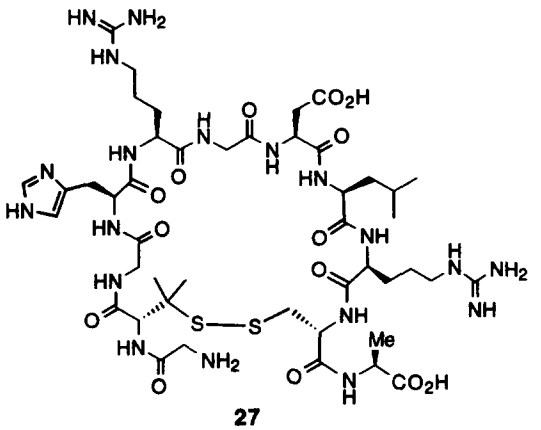
The disulfide-bridged cyclic RGD peptides described above have been simplified further by replacing the disulfide linkage by a lactam.[110] Thus, **31** was optimized to the simplified cyclohexapeptide, cyclo[RGD(Y-OMe)RE]-NH_2 (**36**, IC_{50} = 0.08 μM, human PRP/ADP).[110] Similar 'sulfur-free' cyclohexapeptides such as cyclo[RGDsFf] (**37**) (s = D-Ser, f = D-Phe), were synthesized based on the modification of G-4120 (**26a**).[111]



21, R = H; **22**, R = 1-Np; **23**, R = Ph; **24**, R = 4-CF_3-C_6H_4-; **25**, *n*-Pr



26a: X_1 = O, X_2 = : (G-4120)
26b: X_1 = : ; X_2 = O



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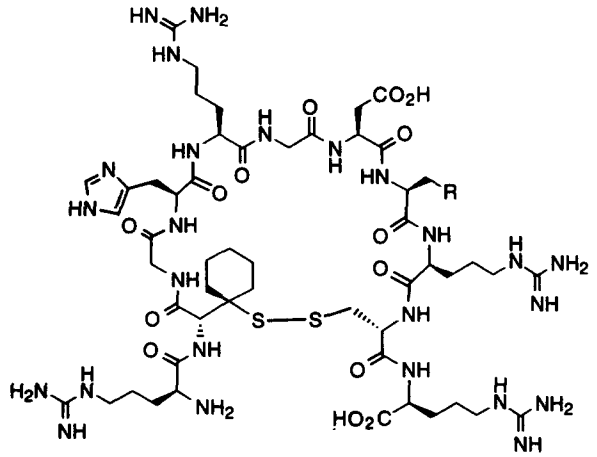
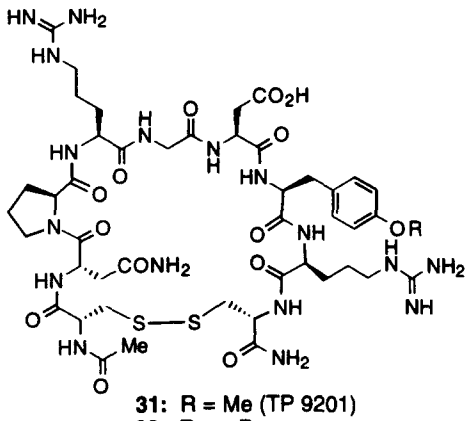


28: R = 4-n-BuO-C_6H_4-
29: R = 4-MeO-C_6H_4-
30: R = 2-Np

Jackson *et al.* have used *m*-(aminomethyl)benzoic acid (Mamb) as the linker to create 'template-constrained' cyclic RGD peptides.[112] For example, a highly potent cyclo[v(N^{α}-Me)RGD-Mamb] (**38**, IC_{50} = 0.02 μM, human PRP/ADP; IC_{50} = 0.006 μM, Fg-GPIIb/IIIa binding) (v = D-Val) has been developed, that bears N^{α}-methylarginine in place of the arginine residue in order to reduce conformational mobility.[112] Methylation at the β-carbon of the aspartic acid residue gives two diastereomers, of which the *S*-diastereomer is 900 times less active than the *R*-isomer (IC_{50} = 0.1 μM, human PRP/ADP).[112] Replacement of the *m*-(aminomethyl)benzoic acid linker by bicyclic linkers, e.g. 8-amino-5,6,7,8-tetrahydro-2-naphthoic acid and 8-(aminomethyl)-2-naphthoic acid, did not improve activity.[112]

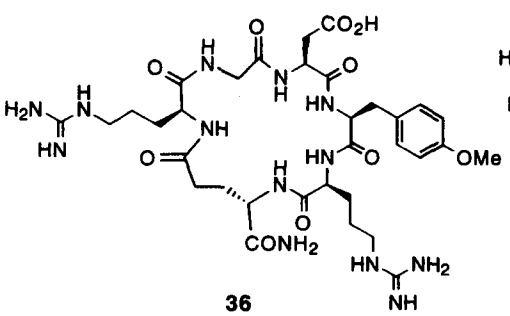


31: R = Me (TP 9201)
32: R = *n*-Bu

A detailed solid and solution state structural study of a family of nine template-constrained cyclic peptides of the general structure cyclo[X-RGD-Mamb][112,113] gave results similar to those reported earlier.[99,100,113] Both X-ray and solution NMR data suggest an extended conformation at the glycine. For example, in cyclo[D-Abu-(N^{α}-Me)RGD-Mamb] (**39**, IC_{50} = 0.02 μM, human PRP/ADP; Abu = 2-aminobutyric acid) a type II' β-turn has been observed, with D-Abu and (N^{α}-Me)Arg at the i+1 and i+2 positions. Glycine exists in an extended conformation, while the Asp residue is at the center of a γ-turn.[113]

Apart from the cup-shaped model (*vide supra*),[57] another model that does not conform to the turn-extended-turn motif has been proposed, that invokes a β-turn at the RGDS segment of cyclo[GRGDSPA].[114] However, a peptide mimic built on a steroidal scaffold, adopting this conformation, was found to be inactive.[115]

Structure–activity relationships of several cyclopeptides bearing the RGDF sequence have been studied.[116] This study has shown that the ring size and the absolute configuration of the phenylalanine residue exerts a marked influence on the activity.[116] For example, replacement of the phenylalanine residue of the cyclo*penta*peptide cyclo[RGDFP] (IC_{50} = 8.8 μM, human PRP/ADP) or the cyclo*hepta*peptide cyclo[GRGDFPG] (IC_{50} = 3.7 μM,



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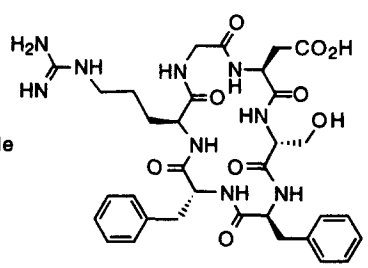
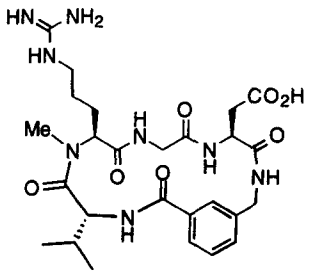


37

human PRP/ADP) with D-phenylalanine results in loss of the activity. However, the stereochemical requirement is completely reversed in the corresponding cyclo*hexa* peptides, i.e., cyclo[RGDfPG] (IC_{50} = 9.2 μM, human PRP/ADP) bearing the D-phenylalanine residue turns out to be active, whereas cyclo[RGDFPG] is inactive.



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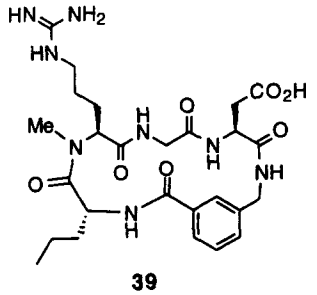

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5. Rational and *de novo* Designs for Non-peptidic RGD Mimetics

5.1. *Peptide mimetics from cyclic RGD peptides*

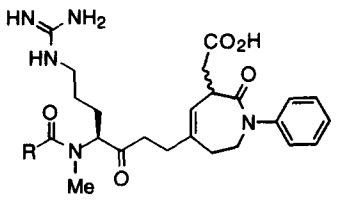
The hypothesis of the turn-extended-turn motif for the RGD sequence has been tested by the synthesis of peptide mimetics of cyclic RGD peptide **15** (*vide supra*) with similar conformational dispositions.[83,102] Thus, peptide mimetics **40** and **41** containing γ-turn elements and a C_7 turn at Asp were designed and synthesized.[102] They bound one order of magnitude more weakly than **15**, yet retained a good level of the activity (IC_{50} of **40** = 1.11 μM, canine PRP/ADP; IC_{50} of **41** = 0.72 μM, canine PRP/ADP). Further optimization, through introduction of a benzodiazepine moiety to mimic the C_7 turn and the extended Gly conformation, an amidinophenyl group to mimic the Arg side chain and a 2-phenylethyl group to mimic a hydrophobic moiety flanking the Asp residue, led to the potent non-peptide mimetic **42** (IC_{50} = 0.15 μM, human PRP/ADP).[83]

The contrasting hypothesis of a cup-shaped conformation for the RGD moiety, i.e., the cup-shaped model, has been examined by benzodiazepinedione-based mimetics which were found to closely mimic the contours of the RGD backbone of G-4120 (**26a**, *vide supra*).[117] In fact, these mimetics, e.g. **43a** (IC_{50} = 0.12 μM, human PRP/ADP) and **43b** (IC_{50} = 0.055 μM, human PRP/ADP), show high activity. The fact that introduction of a planar and more extended disposition by contracting the seven-membered ring diazepinedione skeleton to a six-membered ring led to the loss of activity appears to support the cup-shaped model.[117]

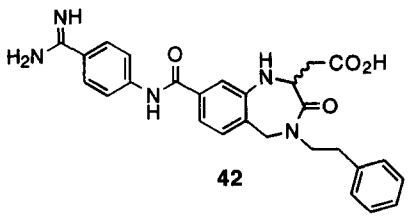
A steroid skeleton has been used to mimic the RGD backbone based on the NMR study of cyclo-GRGDSPA[114] which indicates a β-turn around the RGDS moiety wherein Gly occupies the *i*+1 position. However, the resulting β-turn mimic **44** using allopregnane showed only very weak activity (IC_{50} = 100 μM, human PRP/ADP); this result undermines the validity of the proposed β-turn model.[115]

5.2. *Optimization of lead peptide hybrids and* de novo *leads derived from database*

Following up the development of the *p*-amidinophenyl moiety as a mimic of the arginine side chain in RGD peptides (**6–8**, *vide supra*), Alig *et al.* further modified the C-terminus by using a phenoxyacetic acid moiety as a



40: R = phenyl
41: R = methyl



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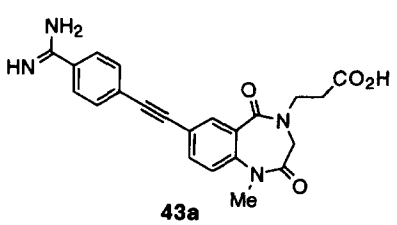
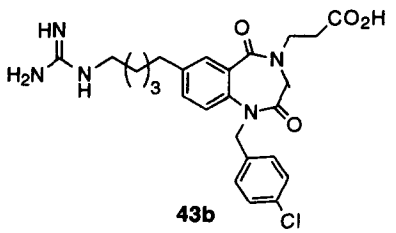
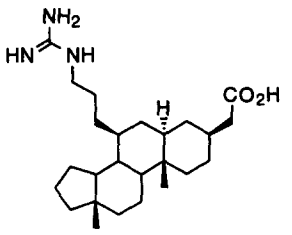


43a



43b



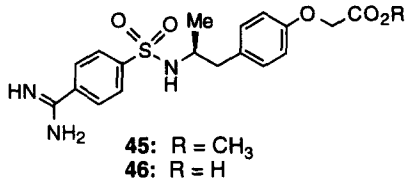
44

surrogate for the side chain of the Asp residue.[92] The importance of the acidic carboxyl terminus and the basic amidino terminus has been established by the comparison of several analogs. The thromboxane A_2 receptor antagonist **45** (IC_{50} = 9.8 μM, human ADP/PRP) containing an ester C-terminus was found to have very weak affinity for GPIIb/IIIa in a solid phase fibrinogen binding assay, despite the fact that **45** can inhibit platelet aggregation induced by different agonists such as ADP, collagen, and thrombin (IC_{50} = 9.8 μM, human ADP/PRP). The corresponding acid analog **46** (IC_{50} = 7.4 μM, human ADP/PRP) showed much higher affinity for GPIIb/IIIa and the structure was further optimized to the highly active **47** (IC_{50} = 0.03 μM, human ADP/PRP).[92] Replacement of the amidino group by a cyano group resulted in loss of activity. It has also been confirmed from the structure–activity relationship study that the distance between the amidino and the carboxyl moieties is critical for activity. The required distance is claimed to be the same as that obtained for RGDS in its extended conformation. Further optimization of **47** by replacing the phenoxyacetic acid residue with a piperidinyloxyacetic acid led to Ro 44-9883 (**48**), which is currently under Phase II clinical trials.[92,118]

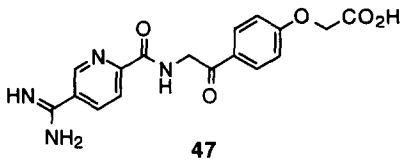
Using a conceptual model for the binding of the RGD unit to GPIIb/IIIa by keeping the distance between the amidino and the carboxyl groups constant, Alig *et al.* demonstrated, for the first time, that the glycine residue was expendable through a 'delink' operation (Scheme 3).[92] The amidino and the carboxyl groups mimicking the Arg and Asp residues, respectively, were linked through a *m*-aminobenzoic acid linker to give a number of active analogs such as **49**.[92]

The peptide hybrid SC-52012 (**6**) is a highly potent GPIIb/IIIa antagonist (IC_{50} = 0.042 μM, human PRP/ADP, *vide supra*). However, the peptidic nature causes **6** to have a very short pharmacodynamic half-life (40 min in the intravenous application to dog). Modification of **6** by replacing the dipeptide portion of the molecule with β-aminopyridinepropanoic acid led to the orally active analog **50** (IC_{50} = 0.12 μM, canine PRP/ADP).[119] Substitution of the pyridine moiety by a phenyl group resulted in a 50-fold loss of activity. Further efforts to increase oral bioavailability have brought about the highly active SC-54701 (**51**, IC_{50} = 0.069 μM, human PRP/ADP), whose prodrug SC-54684 (ethyl ester of **51**) has good oral bioavailability in dog.[120–122]

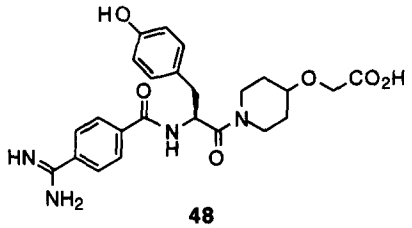
In sharp contrast with these peptide-based rational design approaches represented by Alig *et al.*[92] and Zablocki *et al.*,[91] Hartman, Egbertson and collaborators have undertaken the random screening approach by searching



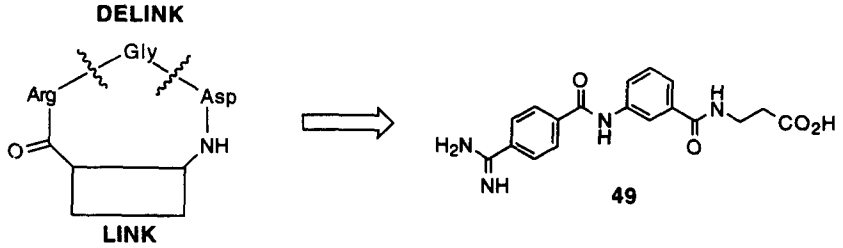
45: R = CH_3
46: R = H



47

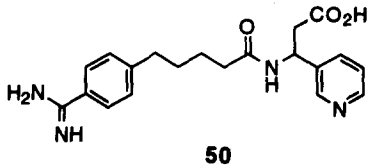


48

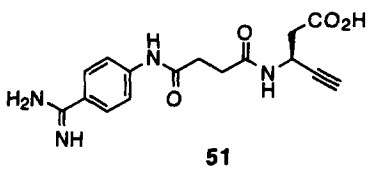


49

Scheme 3. Rationale for the 'delink' operation.[92]



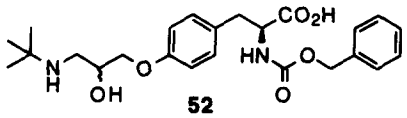
50



51

the Merck Sample Collection for *de novo* leads which have an acidic and a basic moiety in a molecule with the distance between the two functionalities being 10–20 Å.[123,124] This distance requirement was based on the hypothetical spatial separation between the guanidine moiety of Arg and the β-carboxyl moiety of Asp in the RGD sequence. A lead compound **52** (IC_{50} = 26 μM, human GFP/ADP) (GFP = gel-filtered platelets) bearing *N*-Cbz-tyrosine as the key structural component was identified through this screening. Optimization of this lead **52** has brought about a series of highly potent analogs. For example, optimization of the N-terminus group and adjustment of the optimum separation between N- and C-termini led to an active analog **53** (IC_{50} = 0.26 μM human GFP/ADP) bearing piperidine moiety at the N-terminus.[124] Further optimization of the N-substituent of the tyrosine moiety resulted in the discovery of a potent analog **54** (MK-383, IC_{50} = 0.009 μM, human GFP/ADP), the *N-n*-butylsulfonyl derivative of **53**.[124] The *R*-enantiomer of **54** was found to be almost 100-fold less potent. Similarly, the α-methyl-Tyr derivative of **54** was 400 times less active. Thus, it is clear that small changes in this molecule cause inordinately large changes in the activity. Molecular modeling of MK-383 (**54**) in comparison with cyclo-*S,S*-[Ac-CRGDC]-OH (**25**, *vide supra*)[103] indicates that the piperidinyl and the carboxyl moieties of MK-383 (**54**) can act as surrogates of the Arg and Asp side chains.[124] In addition, the ether oxygen may serve as a hydrogen bond acceptor which could explain its high activity.[124] In an attempt to introduce a centrally constraining bicyclic linker to **53**, several RGD mimetics were screened, of which L-709,780 (**55**) was found to possess strong activity (IC_{50} = 0.025 μM, human GFP/ADP).[125]

Several new non-peptidic compounds with relatively rigid structures have been reported as potent inhibitors of platelet aggregation. Examples include a biphenyl derivative **56** (BIBU52, IC_{50} = 0.06 μM, human PRP/ADP) and a cyclic urea derivative **57** (IC_{50} = 0.03 μM, human PRP/ADP) the design of which is based on the hypothetical localized turn in the RGDF backbone.[126] Eldred *et al.* have developed a highly extended piperidine–piperazine-based non-peptide **58a** (GR144053: IC_{50} = 0.037 μM, human GFP/ADP) as an orally active inhibitor of platelet aggregation.[127] GR144053 (**58a**) was developed through the optimization of a lead **58b** (IC_{50} = 54 μM, human GFP/ADP) obtained from a database search, whose parameters were based on a turn-extended-turn hypothesis



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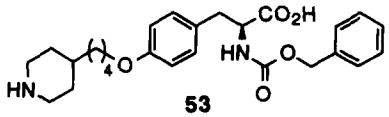

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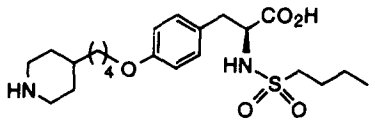
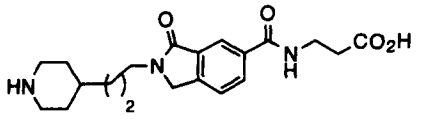


54 (MK-383)



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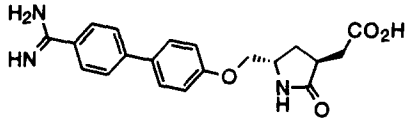

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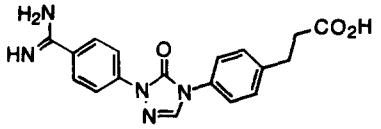

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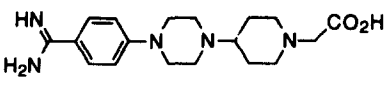
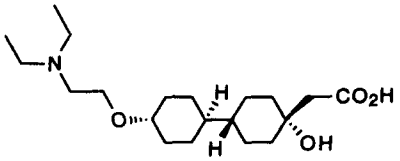


58a (GR144053)



58b

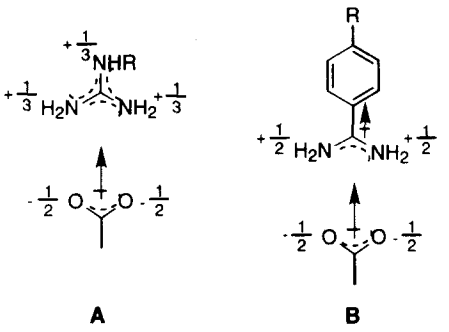
for RGD-containing cyclic semipeptides bearing a conformational disposition similar to that of Peishoff *et al.* [99] GR144053 (**58a**) showed a duration of action of 6.6 h after intravenous administration at 1 mg kg^{-1} and 5.7 h after oral administration at 3 mg kg^{-1} in the marmoset (12 and 8 h, respectively, in the cynomolgus monkey).[128] In an intravenous administration to the dog, GR144053 (**58a**) was shown to prevent coronary thrombosis successfully.[129]

These non-peptide RGD mimetics reflect the current trend towards small and rigid structures in an attempt to develop potential therapeutic agents with good bioavailability. At the same time, these rigid RGD mimetics may contribute significantly to the detailed and fundamental understanding of various interactions between the GPIIb/IIIa receptor and its RGD-based antagonists.

6. Mode of Ligand–Receptor Interaction: Hypothetical Proposals

Based on the structure–activity relationships of a series of RGD peptide hybrids and the presence of a unique DGR sequence (301–303) in GPIIb, [130] Zablocki *et al.* proposed that the carboxylate group of RGDX mimetics, which were assumed to exist as zwitterionic forms, would interact with the guanidinium moiety of the arginine residue in the GPIIb/IIIa receptor through a *reinforced ionic interaction*, i.e., a combination of ionic interaction and hydrogen bonding.[131] This hypothesis is supported by the fact that the substitution of the Asp β-carboxyl group in the RGDS mimetic **6** (*vide supra*) by a tetrazole moiety resulted in complete loss of activity although the replacement of the Phe carboxyl group with the same tetrazole did not significantly affect activity. The tetrazole moiety exists as a negatively charged species in physiological pH interacting in a purely ionic fashion, but is not capable of reinforcing this interaction with hydrogen bonds in contrast to the corresponding carboxylate ion.[131] Computational study in support of this argument has shown that the tetrazole–guanidinium interaction is indeed less favorable than the carboxylate–guanidinium interaction by 4.0–18.9 kcal mol^{-1} (*vide infra*).[131]

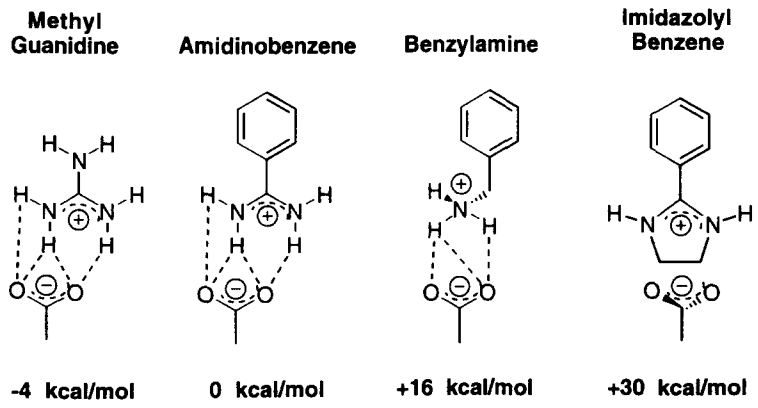
The importance of the reinforced ionic interaction has also been invoked to account for the much greater activity of analogs containing an amidinophenyl moiety than those bearing a guanidine moiety.[91] Zablocki *et al.* proposed that a benzamidinium–carboxylate complex would have more favorable alignment of dipoles than the corresponding guanidinium–carboxylate complex through the reinforced ionic interaction because of the charge localization on two nitrogens in the benzamidinium ion as opposed to three in the guanidinium ion (Scheme 4).[91]



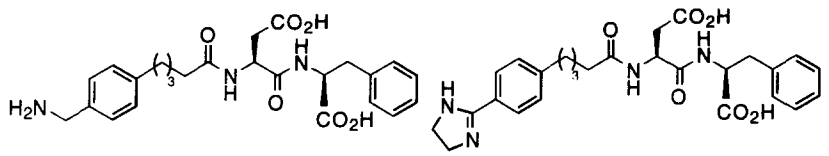
Scheme 4. Reinforced ionic bonding is favored in **B** by a favorable dipole moment alignment.[91]

This hypothesis was further tested by replacing the amidinophenyl group with a benzylamino group (**60**) and an imidazolinophenyl group (**61**). The benzylamine moiety of **60** was anticipated to have a weaker interaction with the receptor than an amidinophenyl group and, indeed, **60** was found to possess only weak activity. The imidazolinophenyl analog **61** was used as a negative test for the reinforced ionic bonding hypothesis, viz., the presence of the ethylene bridge of the imidazole ring in **61** should work against the formation of the planar array of hydrogen bonds that are required for reinforced ionic bonding. In fact, **61** was found to be inactive, lending credence to the reinforced ionic bonding hypothesis. The results of *ab initio* calculations for the ionic complexes of acetate with methylguanidine, amidinobenzene, benzylamine, and imidazolylbenzene as model systems are shown in Scheme 5.[91]

The reinforced ionic interaction hypothesis[91] appears to accurately describe the mode of binding interactions of the



Scheme 5. Relative energy of complexes based on *ab initio* calculations.[91]



60 61

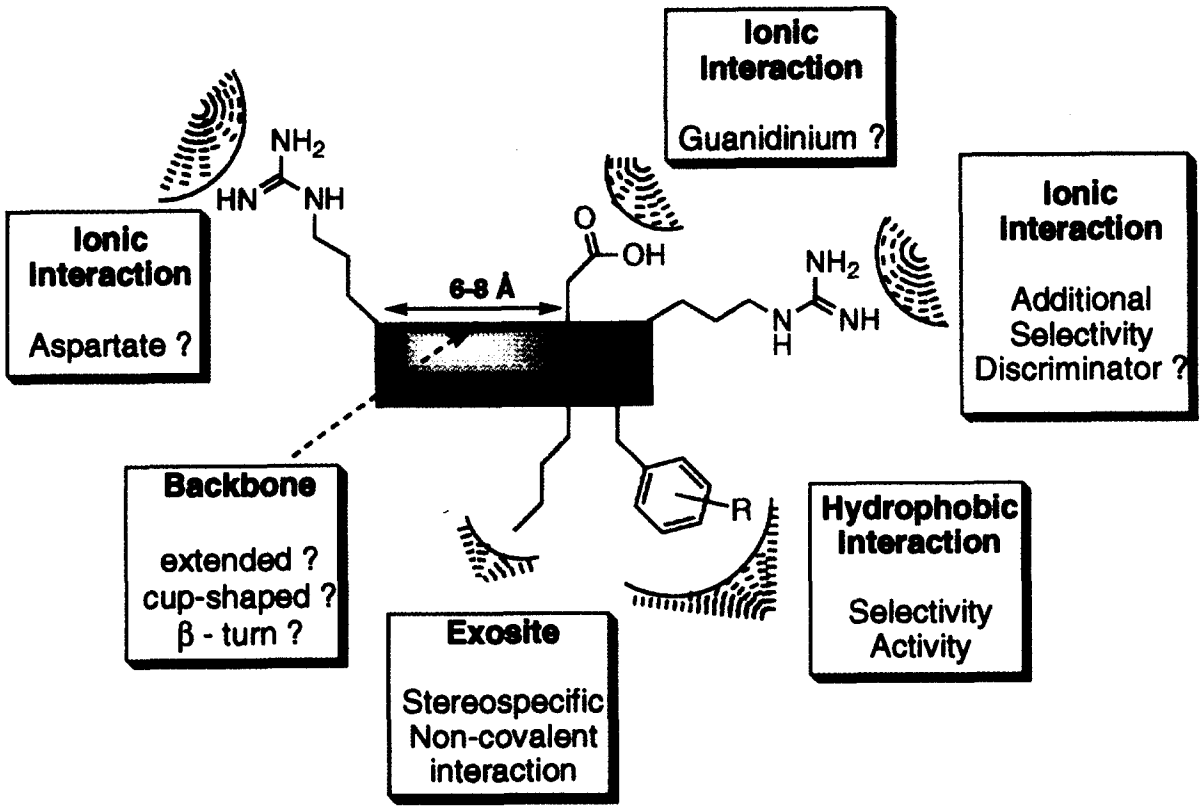
carboxylate and guanidino/amidino functionalities, that are prevalent in most RGD peptides and peptidomimetics, with the GPIIb/IIIa receptor. However, this hypothesis does not explain the high activities observed for MK-383 (**54**) and related analogs (*vide supra*) which possess a 4-piperidinoalkyl moiety as the mimic of the Arg side chain.[123,124]

The fact that the introduction of the *n*-butylsulfonyl group as the N-substituent of the tyrosine residue has brought about a dramatic 27-fold increase in activity from the corresponding N-Cbz analog **53** suggests the existence of a favorable noncovalent interaction occurring at an "exosite" of GPIIb/IIIa, that had not been encountered previously.[123,124] This 'exosite' appears to bind stereospecifically to the molecule since the replacement of the (*S*)-Tyr residue of **54** with (*R*)-Tyr results in a 100-fold loss in the activity (*vide supra*). If the piperidino analog **54** (MK-383) and related analogs are assumed to mimic the RGD sequence, there is apparent inconsistency between this assumption and the proposed reinforced ionic interaction hypothesis. In order to obtain a clear answer for this controversy, further studies are required on the mode of interactions between the GPIIb/IIIa receptor and its antagonists.

Scheme 6 summarizes the modes of binding and conformational requirements for RGD-based antagonists to interact with the platelet receptor, GPIIb/IIIa. Besides the ionic interactions at guanidine and carboxylic acid moieties and the cup-shaped or rather extended backbone conformation of the RGD sequence, the possible existence of an additional hydrophobic binding site in GPIIb/IIIa has been implied by a couple of research groups (*vide supra*)[80–82] although the location of this hydrophobic pocket is still ambiguous. The role of the extra arginine residue flanking the C-terminus of the RGD(Y-OR) sequence of the cyclic peptides **28–32** proposed by Cheng *et al.*[108] (*vide supra*) is also intriguing since this arginine residue appears to increase the receptor binding selectivity of these cyclic RGD peptides to GPIIb/IIIa and shorten the template bleeding time.[109]

7. Selectivity and Efficacy of Platelet Aggregation Inhibitors for Antithrombotic Therapeutics

It has been quite well established that the RGD moiety is a ubiquitous recognition signal for a host of integrin receptors controlling a number of biological functions, several of which perhaps await discovery and full



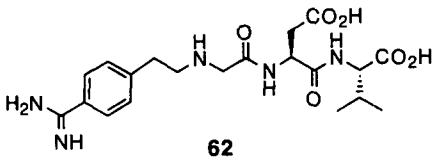
Scheme 6. A representation of the binding modes of RGD-based antagonists to a hypothetical GPIIb/IIIa receptor.

biological characterization. It is also known that the backbone conformations and the nature of flanking residues play an important role in defining the specificity of an RGD-ligand to a particular integrin receptor.[57,87,100] Unlike other integrins, the GPIIb/IIIa receptor is very accommodating in its recognition of a variety of glycoproteins, which indicates the relatively flexible nature of the binding site structure. It is also likely that the activated and non-activated forms of the receptor may recognize the RGD-ligand in different conformational dispositions. Although this property may leverage the design of a variety of GPIIb/IIIa antagonists, the same nature may raise serious concern about the specificity of these antagonists to the GPIIb/IIIa receptor in comparison with other integrin receptors. For example, snake venom proteins and other RGD-containing or mimicking GPIIb/IIIa antagonists can inhibit the binding of various adhesive proteins to the GPIIb/IIIa receptors on the platelet surface, but these agents may also block the adhesive functions of other RGD-dependent integrins such as the vitronectin receptor ($\alpha_v\beta_3$ or $\alpha_v\beta_5$) and the fibronectin receptor ($\alpha_5\beta_1$). Accordingly, a small RGD-peptide or its non-peptide mimic, designed to be a GPIIb/IIIa antagonist, could interfere with the function of other crucial integrin receptors; this cross-reaction may cause serious consequences.

7.1. *Structure–receptor selectivity correlations of RGD peptides and their mimetics*

One of the first studies of the receptor selectivity was carried out by Alig *et al.*,[92] who reported that the replacement of the Arg residue of RGDV (IC_{50} = 127 μM, human PRP/ADP; selectivity = 1.05, GPIIb/IIIa/$\alpha_v\beta_3$) with an amidinobenzyl group brought about a remarkable increase in the receptor selectivity, coupled with a significant increase in the inhibitory potency for platelet aggregation (**62**, IC_{50} = 2.6 μM, human PRP/ADP; receptor selectivity, GPIIb/IIIa/$\alpha_v\beta_3$ = 5.3 × 10^5).[92] Non-peptide inhibitors (**47** and **48**) containing amidinobenzyl group (*vide supra*) have also been reported to possess a high selectivity for GPIIb/IIIa in comparison with $\alpha_v\beta_3$. These results imply the importance of the amidinobenzyl moiety for RGD peptide mimetics to attain a high receptor selectivity for GPIIb/IIIa.

Scarborough *et al.* performed the screening of 62 snake venoms and found that only one of them, obtained from the southeastern pigmy rattlesnake, *Sistrurus m. barbouri*, showed high selectivity to GPIIb/IIIa, i.e., all other snake venoms were non-selective for GPIIb/IIIa.[58] The responsible peptide, barbourin, is very similar to the GPIIb/IIIa antagonists isolated from viper venom, except for the substitution of the RGD sequence by KGD sequence (Scheme 7).[58] It has been shown that barbourin and other KGD-containing analogs have no effect on the interaction of M21 melanoma cell adhesion with vitronectin at micromolar levels. In contrast to this, the RGD-containing venom peptides, echistatin and eristicophin, inhibit the same receptor–ligand interactions at nanomolar levels. In terms of platelet aggregation, barbourin (IC_{50} = 0.3 μM, human PRP/ADP) is only two-fold less active than echistatin.[58]



62

It should be noted that, barbourin (with KGDW sequence) and two other venom peptides, tergeminin and eristicophin (with RGDW sequences), have a tryptophan residue immediately following the K(R)GD sequences (Scheme 7).[58] All of them are weak inhibitors of fibronectin binding to the $\alpha_5\beta_1$ receptor (IC_{50} > 600 nM), whereas echistatin, which has an RGDD sequence, possesses an extremely high potency (IC_{50} = 1–2 nM).[58] It appears that the tryptophan residue prevents these peptides from favorable interactions with $\alpha_5\beta_1$. This observation reinforces the importance of a hydrophobic amino acid residue flanking the C-terminus of the RGD sequence, which has been discussed earlier (see Scheme 6).

Linear and *S,S*-bridged cyclic peptides bearing the XGDW sequence were investigated for their selectivities of binding to different integrins.[132,133] When X was a residue bearing a guanidino moiety such as Arg and guanidinovaleryl, those peptides showed only limited selectivity for the GPIIb/IIIa receptor of platelets over other adhesive protein receptors present in human umbilical vein endothelial cells (HUVEC) including the receptors for vitronectin, fibronectin and fibrinogen.[132] However, the peptides bearing a primary amine as a guanidinyl substitute exhibited enhanced platelet aggregation inhibitory potency with good GPIIb/IIIa selectivity.[132] The increase in the ligand selectivity for

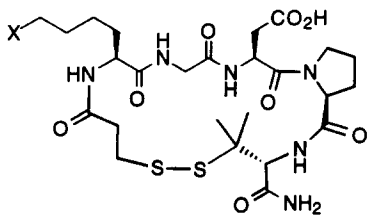
Barbourin	EAGEEDCGSPENPCCDAATCKLRPGAQCADGLCCDQ**CRFMKK**GTVCRV **CRV AKGDW**NDDT **CTGQSA DCPRN**GLY**G**
Tergemin	EAGEEDCGSPANPCCDAATCKLRPGAQCADGLCCDQ**CRFMKK**GTVCRV **CRV ARGDW**NDDT **CTGQSA DCPRN**GLY**G**
Eristicophin	ZRQEEPCATGPC**CRRCKF**KRAGKV **CRV ARGDW**NNDY **CTGKSC DCPRN**PWN**G**
Echistatin	ECESGPC **CRNCKF**LKEGTI **CKR ARGD**DMDDV **CNGKTC DCPRN**PHK**GPA**

Scheme 7. Amino acid sequence homology for barbourin and other snake venom derived disintegrins.

GPIIb/IIIa was also observed through the N^{α}-acylation of the lysine residue of tetrapeptide KGDW with a piperidine-4-carbonyl group, that increased the inhibitory activity for platelet aggregation as well.[133]

It is worth mentioning that MK-383 (**54**) (*vide supra*) is a selective antagonist of platelet receptor (GPIIb/IIIa)–fibrinogen association as compared to fibrinogen binding to the HUVEC receptor. This selectivity makes a sharp contrast to RGDW and echistatin, which are non-selective and have a preference to the HUVEC receptors rather than the platelet receptor.[124]

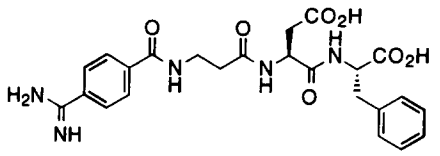
Incorporation of the KGD sequence into a disulfide-bridged cyclic peptide scaffold, followed by optimization has led to cyclo-*S,S*-[MprKGDWPPen]-NH_2 (**63**, IC_{50} = 1.2 μM, human PRP/ADP) and its analogs **64** (IC_{50} = 0.08 μM, human PRP/ADP), **65** (IC_{50} = 0.3 μM, human PRP/ADP), and **66** (IC_{50} = 0.45 μM, human PRP/ADP).[59] These highly active GPIIb/IIIa antagonists are virtually inactive for the vitronectin receptor ($\alpha_v\beta_3$) even at relatively high concentrations (20 μM).[59] It is noteworthy that the substitution of the lysine residue of **63** with homoarginine greatly increases the inhibitory potency (>10-fold) for platelet aggregation while the resulting analog **64** retains excellent receptor selectivity.[59] These cyclic peptides are equivalent to barbourin with respect to their potency as well as their specificity to GPIIb/IIIa. The pK_a value of the amine functionality also appears to be crucial in determining the receptor specificity to GPIIb/IIIa besides the conformation and length of the basic side chain. The lysine and imidated-lysine analogs (**63**, **65**, **66**), that are less basic than the guanidine-containing analogs, are even more specific for GPIIb/IIIa than the homoarginine analog (**64**) despite the fact that these side chains have an identical number of methylenes.[59] Thus, the highest receptor specificity is achieved by the Lys and imidated-Lys analogs while the Arg-containing analogs are the least specific.



63: X = NH_2-
64: X = $H_2NC(=NH)NH$-
65: X = $CH_3C(=NH)NH$-
66: X = $PhC(=NH)NH$-

7.2. *Conformational states of GPIIb/IIIa: ligand-induced binding sites and agonism of GPIIb/IIIa with RGD peptides and mimetics*

The functions of the GPIIb/IIIa receptor in platelet are believed to be dependent on its conformations. For example, the binding site of GPIIb/IIIa for soluble fibrinogen is hidden under normal physiological conditions, while the activation of the receptor induces a conformational change leading to the exposure of the fibrinogen-binding epitope. The outside-in signaling events that follow the ligand-binding may also be transmitted through conformational changes in the receptor complex.[43] The binding of fibrinogen to the activated GPIIb/IIIa receptor is followed by the expression of certain epitopes which are called 'ligand-induced binding sites (LIBS)'.[134] While the physiological role of LIBS is still uncertain, recent studies on LIBS using monoclonal antibodies indicated a possible role of LIBS in the regulation of platelet function.[134,135] In contrast to fibrinogen, it has been shown that RGD peptides and their mimetics are capable of binding to the inactive conformation, i.e., 'resting state', of GPIIb/IIIa, but they may also cause the exposure of LIBS[95,136] and even the partial agonism of receptor function in purified GPIIb/IIIa.[137] For instance, RGDV and Ro 43-5054 (**67**, IC_{50} = 0.05 μM, human PRP/ADP)[95] create LIBS in purified GPIIb/IIIa, that is maintained even after the ligand is removed by gel-filtration.[136] However, Ro 44-9883 (**48**) has been shown not to activate the fibrinogen-binding function of GPIIb/IIIa after removal, i.e. **48** does not express LIBS.[59,136] At present it is not known whether the continuous exposure of LIBS in the long-term administration of an orally active RGD-based inhibitor might lead to antigenic response or other consequences or not. Thus, this question should be answered in the future.



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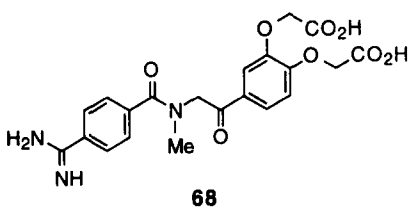
7.3. *Bleeding and thrombocytopenia — preclinical and clinical studies*

Hemorrhagic events and thrombocytopenia are likely side effects accompanying the inhibition of platelet function with antithrombotic agents. Template bleeding time (based on the time required for cessation of bleeding from a uniform cut in the skin) has been extensively used to demonstrate the hemorrhagic potential of antithrombotic drugs. It must be noted, however, that this measure is highly variable in terms of species used, sites of injury and several other experimental factors. As discussed earlier in relation to monoclonal antibodies, it has also been established that bleeding times do not necessarily correlate with actual hemorrhagic events in a clinical setting.[55,56] Therefore, the results of bleeding time experiments should be interpreted cautiously. In a setting where an acute reversible antithrombotic is used, the risk of spontaneous hemorrhage based on bleeding time prolongation is minimal. However, a more accurate risk assessment may be required when the antithrombotic is administered over extended periods of time.

In a comparative study on the ability of TP9201 (**31**) and G4120 (**26a**) to avert thrombosis in a hamster model,

TP9201 (**31**) did not show significantly prolonged template bleeding time at effective doses.[138] In contrast, G4120 (**26a**) caused an increase in template bleeding time from 1.3 to 12 min even at a sub-effective dose. In a pig carotid thrombosis model,[139] both Ro 44-9883 (**48**) and G4120 (**26a**) showed doubling of the bleeding time at the minimum dose required for complete inhibition of thrombotic action. At higher doses, G4120 (**26a**) caused rather severe bleeding time prolongations (> 30 min).[109]

A comparison of Ro 43-8857 (**68**) and MK-383 (= L-700,462) (**54**) in guinea-pig models has revealed that Ro 43-8857 (**68**) increases bleeding time only at supra-maximal doses with respect to inhibition of platelet aggregation.[140] The result for Ro 43-8857 (**68**) suggests that the antiaggregatory effect and the prolongation of bleeding time associated with antithrombotic agents could be dissociated. The same study has shown that MK-383 (**54**) (duration of action ~ 1 h), which is not as long lasting as Ro 43-8857 (**68**) (duration of action ~ 5 h), causes an increase in template bleeding time.[140] In a dog model, however, both compounds led to longer bleeding time.[140] These results reflect the differences in species-specifity of these two agents although the mechanism of action for these two agents are likely to be the same, and also clearly indicate the necessity for careful interpretation and comparison of data obtained from different animal models as well as cautious extrapolation of those data to clinical applications to humans.[140,141]



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The clinical evaluations of synthetic RGD-peptides and their mimetics are still few in number compared to the data available for monoclonal antibodies. The Phase I clinical trials of MK-383 (**54**) in healthy human volunteers for *ex vivo* platelet aggregation and template bleeding time have revealed an increase in bleeding time from 5 min to 22.7 min in a 1-h infusion study at 0.4 $\mu g\ kg^{-1}\ min^{-1}$, which returns to normal after 3 h[142] where the effectiveness in controlling *ex vivo* platelet aggregation lasts for a period of about 3 h. Similar results were obtained in a 4 h infusion study at 0.1–0.2 $\mu g\ kg^{-1}\ min^{-1}$, with increases in template bleeding time which remained slightly above normal even 3 h after infusion.[142] Similar studies on Ro 44-9883 (**48**) on healthy human volunteers revealed a two-fold increase in template bleeding at a dose effecting complete inhibition of thrombosis.[118]

8. Conclusion

This review has covered several aspects of molecular design that have led to the development of highly potent and receptor-specific RGD-mimetics as potential antithrombotic therapeutics. The development of antithrombotic drugs with much greater therapeutic scope than currently available treatments is an attractive proposition considering the widespread prevalence of cardiovascular and cerebrovascular disease. The inhibition of fibrinogen-mediated platelet aggregation has proved to be a successful strategy in the development of monoclonal antibodies and drug candidates based on the RGD sequence as potential antithrombotics. Recent Phase III clinical trials on the monoclonal antibody, c7E3 Fab (CentoRx), have confirmed the applicability of these new drug candidates as acute antithrombotics. The development of orally active and long-lasting RGD-mimetics is currently in progress and should eventually allow a chronic treatment of thrombotic disorders.

Although the three-dimensional structure of the receptor binding site has not been determined, systematic and rational design of highly active ligands to the receptor has disclosed critical functional as well as spatial requirements for favorable ionic and hydrophobic interactions at the receptor site. Molecular modeling has been instrumental in improving the understanding of the optimal three-dimensional structure of an RGD peptide or its mimetic required for high potency and receptor specificity.

The use of RGD-mimetics as antithrombotics presents several attractive features. For instance, it is possible to tailor the RGD moiety systematically to obtain peptidomimetics with variable physiological half-lives. The spectrum of physiological stability of these agents can be exploited for particular medical needs. This feature allows RGD-mimetics to be useful in both acute and chronic situations. It has been demonstrated that potential problems such as immunogenicity, extended bleeding times and thrombocytopenia can be circumvented, to a large extent, by careful design of RGD-mimetics. The specificity of RGD-mimetics for the GPIIb/IIIa receptor can also be attained as evidenced by the development of several highly GPIIb/IIIa-specific peptide mimetics. RGD-based drugs can prove to be cheaper, more readily available and, perhaps, safer alternatives to monoclonal antibodies. Some of these RGD-mimetics are currently under clinical trials as acute antithrombotics (intravenous administration).[91,92,118,142]

Finally, the current trend in the development of RGD-mimetics is clearly directed towards orally active and safe antithrombotic drug candidates.[119] It should be noted that the use of orally active and long-lasting antithrombotics requires special caution in terms of chronic toxicity and possible hemorrhagic consequences. These drugs will be particularly useful for the treatment of patients with recurrent unstable angina or for the prevention of recurrence of thrombosis over a long period of time in cases of myocardial infarction and strokes. Several RGD-mimetics are being evaluated for their oral activity in animal models,[120–122,128,140] and some of those will go into human clinical trials in the near future. It is expected that newer and more effective RGD-mimetics will be developed, which will eventually serve as powerful therapeutic antithrombotic drugs to save many lives.

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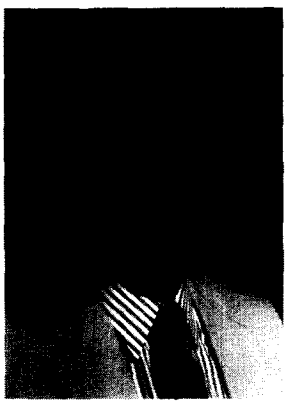
Biographical Sketch



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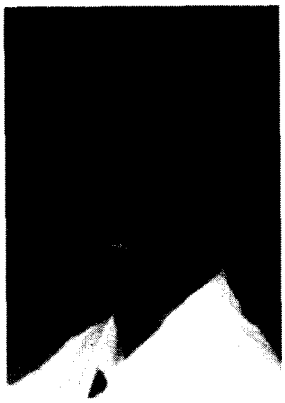

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