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Artificial RGD receptor molecules

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Integrins play a pivotal role in cell–cell adhesion, signalling and apoptosis. Many extracellular proteins use the RGD sequence (arginine–glycine–aspartate) as a key to dock onto and unlock their respective binding partners at the cell membrane ($\alpha_V\beta_3$-, $a_{IIb}\beta_3$- and $a_5\beta_1$-integrin). Here, the RGD signal is transduced into the cytoplasm and triggers a variety of biological events such as blood coagulation, cell–matrix binding, cell differentiation and angiogenesis. A misfunction of this recognition system causes severe diseases, rendering the RGD recognition system an attractive drug target. Inhibition of RGD–integrin interactions can be reached in two different ways, by blocking integrins with RGD mimetics or by capping RGD-containing proteins by artificial RGD receptors. This review provides an overview over the very young history of artificial RGD receptor development, beginning with early research in arginine recognition, over the discovery of the first primitive RGD receptor until the present state of research and future prospects.

Keywords: RGD peptide; integrins; molecular recognition; artificial receptors; amino acids

Introduction

Integrins represent the major class of biological receptors, which mediate cell–cell and cell–matrix interactions (*1*). They are heterodimeric glycoproteins, located inside the cell membrane and present the head portions of both subunits to the extracellular space. Cell recognition takes place when specific extracellular proteins dock onto the cell membrane and recognise a specific integrin type. This recognition process hinges on a small key peptide sequence, presented on a solvent-exposed loop of all approaching extracellular proteins. It contains three consecutive critical amino acids: arginine–glycine–aspartate and is, therefore, generally coined as RGD motif. This tripeptide sequence literally serves as a key which can be inserted into the integrin to lock and open it.

In 1984, it was discovered that the RGD sequence mediates cell adhesion by fibronectin (*2*); subsequent findings quickly demonstrated that the RGD sequence is a general powerful recognition motif utilised by many other proteins such as vitronectin, laminin or the von Willebrand factor, which all play a pivotal role in cell adhesion biology. Fundamental biological processes such as cell differentiation, signalling and apoptosis depend on the interplay between RGD-containing proteins and their related integrin receptors (*3–5*). The integrin family is highly variable because its members differ in their composition through the combination of eight different α- and β-subunits. A single integrin is able to discriminate between the RGD sequences of different proteins by two main mechanisms: it recognises not only the specific chemical nature of these three amino acids but also the specific conformation of each of these individual RGD loops. The relative orientation of the alkylguanidinium and the alkylcarboxylate side chains can adopt all transitions from a linear, fully extended conformation up to a tightly folded cyclic loop. In some cases, the integrin also interacts with the fourth amino acid residue, e.g. in the RGDS sequence (arginine–glycine–aspartate–serine) typical for fibronectin.

Dysfunction in this critical recognition system can cause pathogenic effects such as tumour-induced angiogenesis, osteoporosis, myocardial infarction or thrombosis (*6–8*). The resulting severe diseases reveal the medicinal and pharmaceutical importance of the RGD recognition system and render it an attractive drug target. A precise control over pathologic RGD recognition events by externally added small molecules would not only help to better understand this important natural signalling pathway but also have immediate therapeutic relevance. In principle, inhibition of the RGD–integrin interaction can be reached in two different ways. One has been pursued intensively by pharmaceutical research; the other – more difficult one – has been totally neglected. A great deal of synthetic, computational and biological effort has been devoted to develop the so-called *RGD mimetics*, which imitate polar groups and their relative orientations in a specific RGD sequence, in order to compete with the native ligands of such integrins (Figure 1(a) and (b)).

Numerous academic and industrial groups have developed peptide-derived or non-peptidic RGD mimetics,

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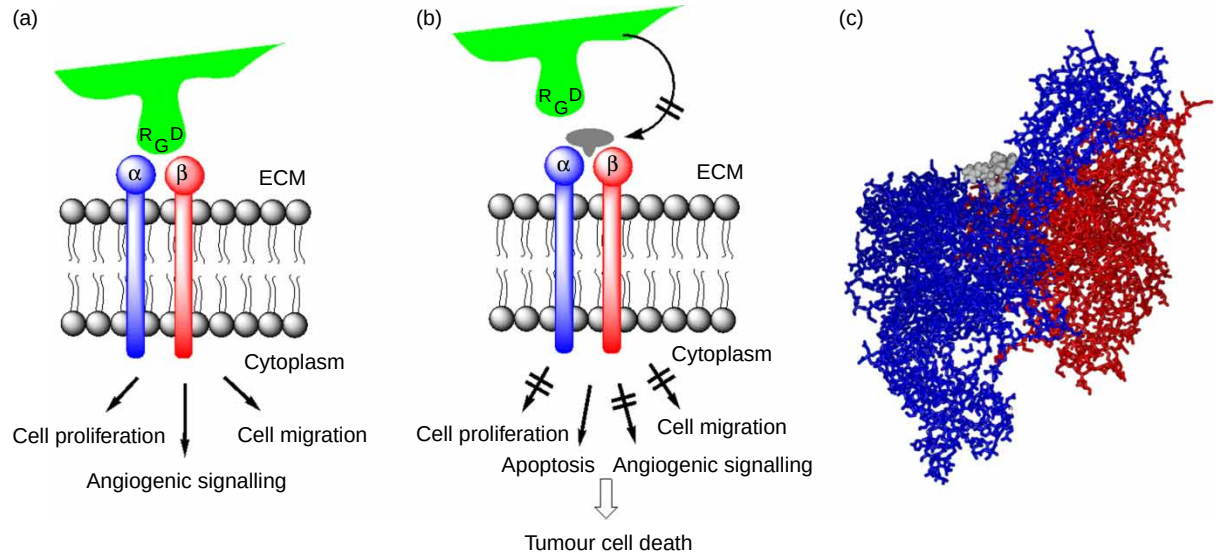


Figure 1. (a) Schematic of the integrin–RGD 'lock and key' principle: an RGD-containing protein (key) docks onto the membrane-spanning α,β-integrin dimer (lock) and induces a biological effect. (b) Conventional interference principle: the ligand binding site on the integrin is blocked by an RGD mimetic (grey). (c) Crystal structure of the cRGDf[NMe]V peptide (grey) in its complex with the α,β-integrin dimer $\alpha_V\beta_3$ (blue–red).

most of which showed some biological activity. Most notably, an elegant approach was introduced by Kessler et al. (*9*) who synthesised a series of RGD-containing cyclopeptides with five- and six-membered rings containing single D-amino acid residues. In extended NMR spectroscopic studies, the structural principles governing the formation of preferred fixed conformations were unravelled and used for a conformational design of integrin-specific RGD mimetics, which showed promising *in vitro* biological effects. During the course of these studies, a whole set of cyclic hexapeptides and pentapeptides was synthesised and thermodynamic equilibria as well as dynamics of their 3D structures in solution were analysed by 2D NMR techniques.

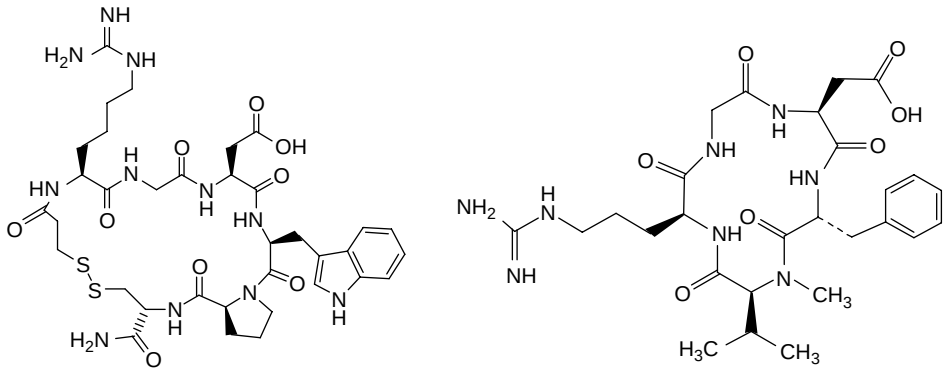
Finally, systematic tuning of conformational restrictions accompanied by MD simulations and subsequent bioassays culminated in the preparation of the strong lead compound *cyclo*-RGDf[NMe]V (Figure 2), a powerful integrin antagonist, which effectively inhibits tumour-associated angiogenesis (*9–11*). This very small cyclopeptide, which is currently undergoing clinical trials, imitates the RGD loop in vitronectin. In 2002, Arnaout et al. (*12*) gained a crystal structure of this cyclopeptide in its complex with integrin $\alpha_V\beta_3$, the only crystal structure of an RGD–integrin complex which is available today. This structure reveals that the peptide is inserted in a cleft between the so-called β-propeller from the α_V-unit and βA domains from the β_3-unit on the integrin head. As expected, almost all peptide–protein contacts are established by the RGD sequence (Figure 1(c)) (*12*).

Figure 2 displays some related RGD mimetics, some of which have already been approved as drugs. Eptifibatide, a somewhat more flexible cyclohexapeptide with an internal disulphide bond, inhibits integrin $\alpha_{IIb}\beta_3$. It was developed entirely by pharmaceutical industry and is used against myocardial infarction or in heart surgery. Other potent drugs are drastically simplified and often rigidified RGD mimetics which contain only one stereogenic centre and replace guanidinium ions by (hydroxy)amidines (Figure 2).

Artificial RGD receptor molecules

A completely different approach to block pathologic RGD–integrin interactions is to construct integrin mimetics, which recognise RGD-containing proteins. In principle, this simplifies the task of developing a small artificial host molecule, which can serve as a competitive 'lock' for the RGD sequence in native proteins. Due to the encapsulated RGD 'key', the whole extracellular protein is not able to bind anymore to its respective target integrin (Figure 3(a)). However, since the RGD sequence exists in a variety of different conformations, depending on the parent protein, it is mandatory to provide synthetic hosts, which can distinguish between them (Figure 3(b)).

Some years ago, the Schrader and Schmuck groups embarked on a programme to pursue this alternative approach towards conformation-specific RGD host molecules. The starting point was marked by the discovery that *m*-xylylene bisphosphonates **1** bind guanidinium cations in a chelate-type manner reminiscent to the natural diaspartate binding site found in the crystal structure of the cRGDf[NMe]V–$\alpha_V\beta_3$ complex. In both cases, a bidentate salt bridge with a network of ion-pair-reinforced hydrogen



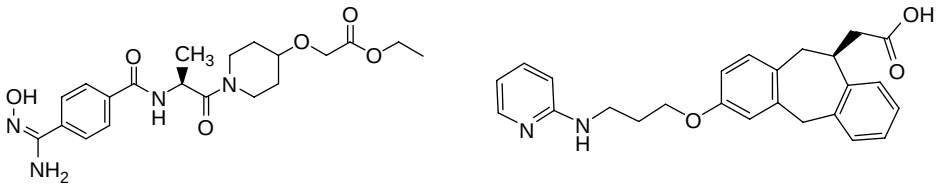


Figure 2. Synthetic RGD mimetics: cRGDf[NMe]V (cilengitide, Merck KGaA (Darmstadt, Germany), specific for $\alpha_V\beta_3$-integrin); eptifibatide (Integrilin®, Millennium Pharmaceuticals (Cambridge, MA, USA) GlaxoSmithKline (Brentford, UK)/Schering-Plough (Kenilworth, NJ, USA), specific for $\alpha_{IIb}\beta_3$-integrin); sibrafiban (Hoffmann-La Roche (Basel, Switzerland), specific for $\alpha_{IIb}\beta_3$-integrin); SB-265123 (GlaxoSmithKline, specific for $\alpha_V\beta_3$-integrin).

bonds firmly embraces arginine's guanidinium cation and holds it in place (Figure 3(c) and (d)) (*13*).

An early screening experiment for RGD binders was carried out by Ruoslahti et al. (*16*) In 1999, a patent was filed which announced the discovery that cyclic peptides with the consensus sequence (W/P)DD(G/L)(W/L)(W/L/M) bind to fibronectin and related RGD-containing proteins. The best candidate was *cyclo*-CWDDGWLC **2**, with an internal disulphide bond, which is depicted in Figure 4(a). It was isolated from a library of short cyclic peptides and peptidomimetics in the course of specific assays for RGD-binding activity. The peptides were presented in the form of a phage library, which was used to isolate minimal receptor sequences that bind to fibronectin in affinity panning. Thus, the cyclic octapeptide *c*-CWDDGWLC was identified as the most potent inhibitor for RGD-dependent cell attachment to fibronectin and vitronectin. Fibronectin itself tightly binds to a *c*-CWDDGWLC affinity column and could subsequently be re-eluted with an RGD-containing peptide. Interestingly, a specific region within the β-subunit of RGD-binding integrins had previously been shown by site-directed mutagenesis to be involved in ligand binding (*14*), which includes the short peptide stretch KDDLW – quite similar to the above-described sequence. Short synthetic peptides corresponding to this integrin region bind to the RGD-containing protein, but their affinities were markedly reduced if both aspartate residues are mutated to alanines. In summary, the combination of phage display technology, rational design and mutagenesis has lead to structural integrin mimetics, which can significantly reduce the cell attachment of an RGD-containing protein (*15*, *16*). However, these are still sensitive to proteolytic degradation.

In 2009, Liakopoulou-Kyriakides et al. (*17*) tried to overcome this drawback by employing the molecular imprinting technology (Figure 4(b)) in order to generate a new chromatography stationary phase for the separation of RGD peptides from mixtures. They used the free RGD peptide as template molecule and prepared molecularly imprinted polymers from only two functional monomers, i.e. methacrylic acid (MAA) and acrylamide (AA). Three different crosslinking monomers were tried with trimethylpropane trimethacrylate producing the best results in terms of RGD selectivity. MAA was later postulated to form interactions with arginine, while AA's amide is both a moderate hydrogen bond donor and acceptor. To establish RGD selectivity, four other peptides were used in rebinding experiments, namely KGD (lysine–glycine–aspartate) and three other completely different peptides. Selectivity factor values ranged from 1.27 to 1.98, net rebinding values for RGD and KGD were similar (~8–10%), while those for other peptides remained very small (2–4%). These data indicate low affinity and only moderate selectivity for the

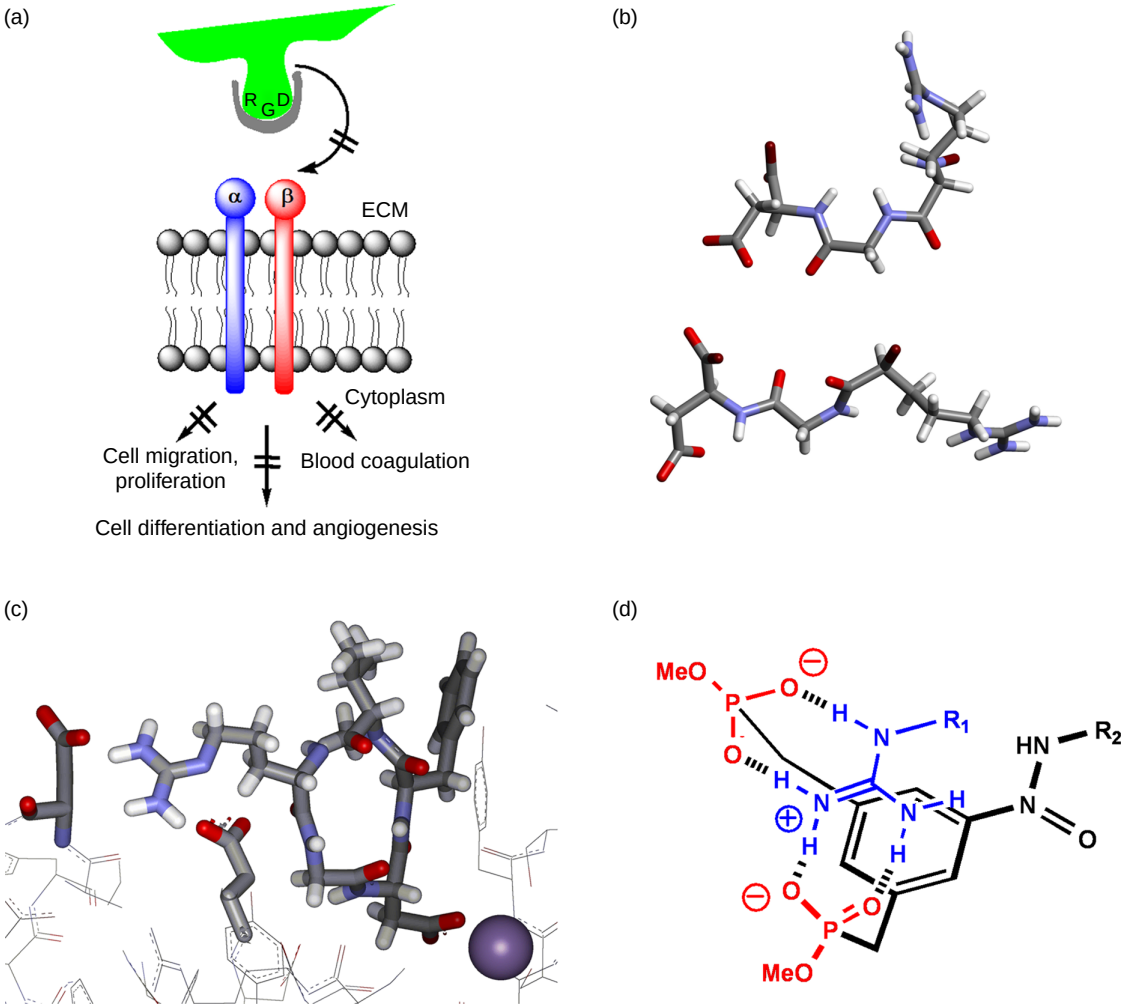


Figure 3. (a) Alternative concept for RGD–integrin interruption: RGD-capping by an artificial RGD receptor molecule (grey) prevents docking onto the membrane-bound integrin (blue–red); (b) folded and extended conformation found in the RGD loops of fibronectin and vitronectin (from crystal structures); (c) chelate-type binding motif from the crystal structure of the complex between cRGDf[NMe]V and $\alpha_V\beta_3$ and (d) chelate-type binding motif in a *m*-xylylene bisphosphonate–arginine complex.

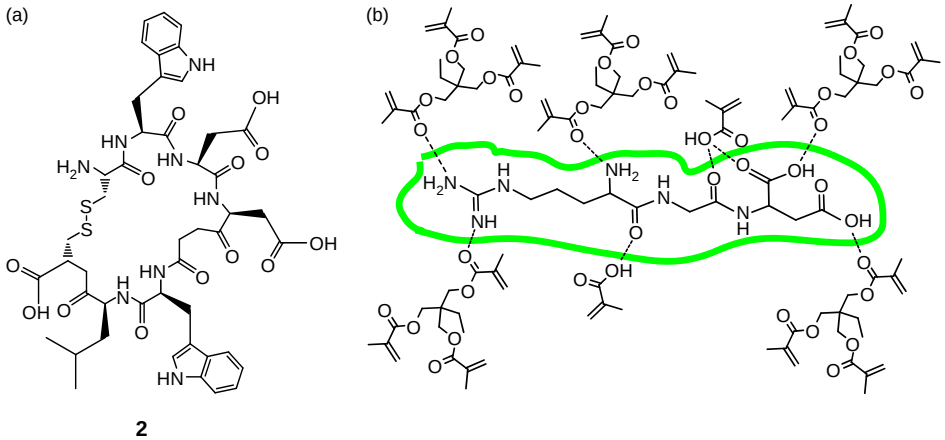


Figure 4. (a) Lead candidate *cyclo*-CWDDGWLC, identified from phage-display assays as a general RGD protein binder and (b) attempted molecular imprinting for the free RGD template peptide: proposed non-covalent interactions between functional monomer, crosslinker and template.

template molecule. In addition, the free RGD peptide was examined in methanol solution, where K_d values were in the high micromolar range (*17*).

Surprisingly, to the best of our knowledge, apart from our own project, these are the only artificial RGD receptor molecules published to date. We tentatively explain this with a lack of powerful concepts for the generation of water-soluble host molecules for zwitterions in physiological solution. In the aqueous medium, hydrogen bonds must be reinforced with electrostatic interactions and complemented by hydrophobic forces to achieve high affinities. Zwitterions are prone to undergo intramolecular as well as intermolecular self-association, which often renders the desired host–guest complexes insoluble in buffered aqueous solution – especially in the presence of high salt loads.

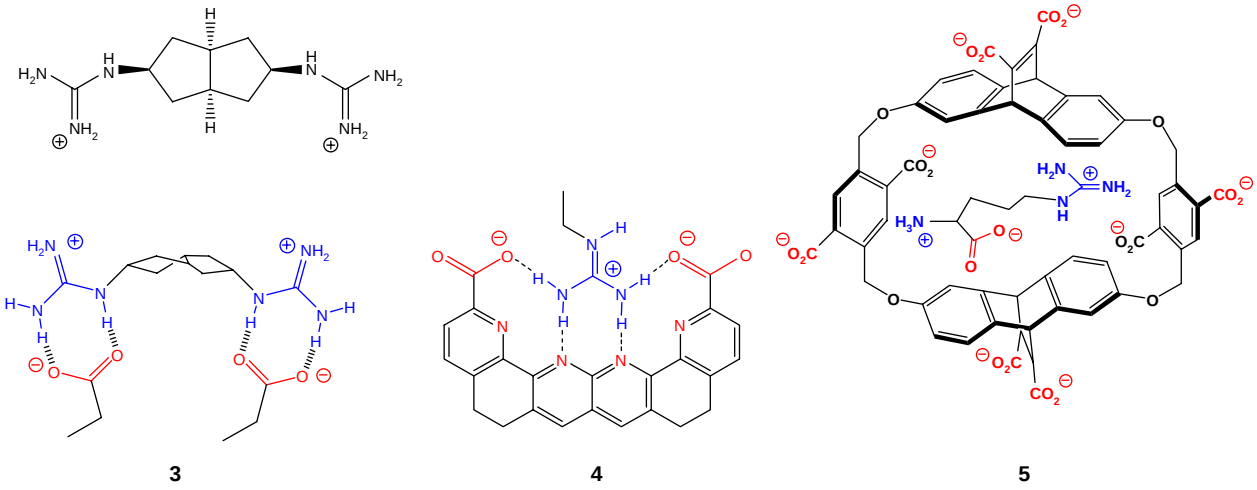
For isolated arginine and aspartate as well as for model peptides containing only anionic or cationic residues, some powerful receptor molecules have been developed. Hamilton et al. (*18*) created a rigid concave scaffold **3** with two pre-organised guanidinium moieties (Figure 5 (a)), which specifically bind two aspartate carboxylates in the $i + 3$-relation of an α-helix. Small aspartate-containing model peptides are bound with millimolar affinities, albeit only in a 10% water–methanol mixture (*18*).

Bell et al. (*19*) synthesised crescent-shaped molecule **4** with multiple annulated pyridine and dihydrobenzene rings, which they coined as 'the arginine cork' because of its high preference for arginine (Figure 5(b)). It prefers diarginine as its guest and displays K_d values in the low micromolar range for molecular recognition in water. The architectural concept is based on a remarkably rigid host skeleton which leads to a large degree of pre-organisation of hydrogen bond acceptors, reinforced by two strategically placed carboxylates for additional ionic interactions between the dianionic host and the dicationic guest (*19*). Problematic features are limited solubility and difficult synthetic access.

Later the Dougherty group constructed a much larger, highly charged cyclophane receptor (**5**), which is selective for arginine and lysine dipeptides (almost micromolar affinities in aqueous solution, Figure 5(c)). The clue is a combination of cation-π interactions with electron-rich receptor π-faces and multiple salt bridges, complemented by total inclusion of one amino acid inside the receptor cavity, leading to solvent exclusion; the second basic residue remains solvent-exposed (*20*).

In 1997, Schrader (*13*) discovered that the *m*-xylylene bisphosphonate dianion is a powerful guanidinium binder in dipolar aprotic solution (DMSO) and forms a similar arrangement as the well-known Tat–TAR complex between the AIDS virus's RNA fragment (two phosphate groups) and a regulatory protein (arginine, Figure 3(d)) (*14*). This recognition motif prefers delocalised, soft guanidinium cations over hard ammonium ions and especially arginine over lysine residues. Systematic investigations were carried out to study the specific interplay of non-covalent attractive forces with modified model compounds. To this end, additional substituents with varying electronic and steric properties were introduced in the 5-position of the central benzene ring (see selection in Figure 6).

It was assumed that three kinds of non-covalent interactions play a key role in guanidinium recognition: electrostatic and hydrogen bond interactions with both phosphonate moieties and cation–π interactions with the receptor's central aromatic ring, which was placed in direct van der Waals contact by force field calculations. Direct proof of cation–π interactions was provided with small electronics withdrawing and donating groups in 5-position of this central benzene ring; these substituents could not form any additional hydrogen bonds to the



3 **4** **5**

Figure 5. (a) Pre-organised host molecules for aspartate and arginine: Hamilton's concave $i + 3$-diaspartate binder **3**, (b) Bell's planar 'arginine cork' **4** and (c) Dougherty's arginine-cavitand **5** with putative arginine inclusion.

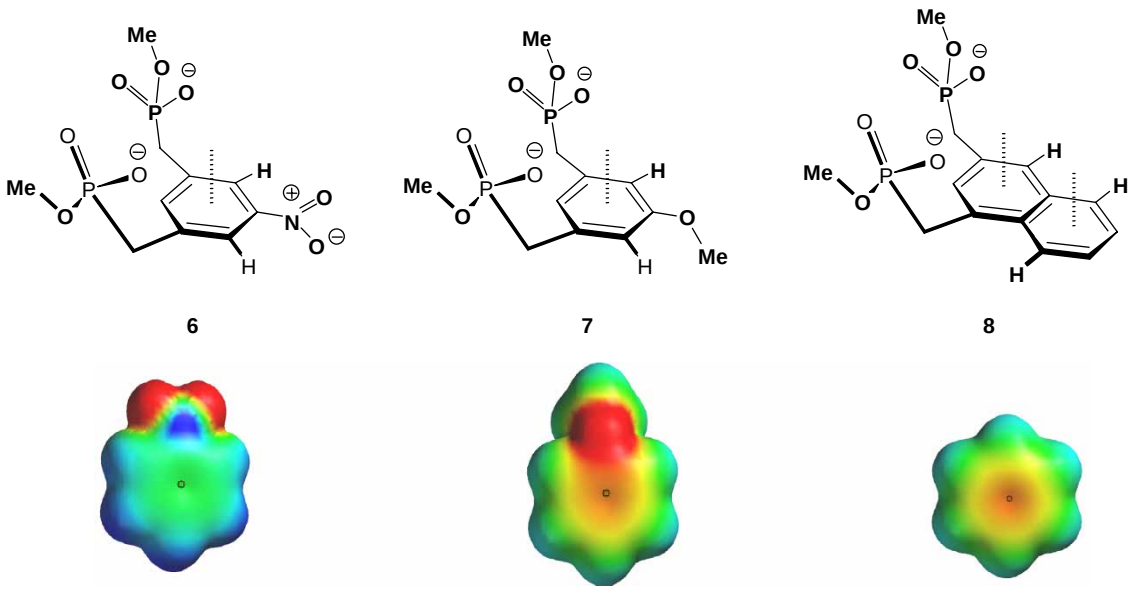


Figure 6. *m*-Xylylene bisphosphonate host molecules **6**–**8** with additional substituents in the 5-position – probing the π-cation interaction. Below: ESP of nitrobenzene, anisole and benzene (AM1 calculations).

guanidinium cation because they were geometrically locked in the aromatic plane of the receptor molecule. Intriguingly, all electronic withdrawing groups lowered and all donating groups raised relative affinities towards methylguanidine hydrochloride, reflecting weakened or strengthened interactions between the guanidinium cation and the aromatic system. A close correlation was found between free-binding enthalpies and the electrostatic surface potential (ESP) of the respective central rings. Dissociation constants could also be lowered by increasing the π-face of the receptor, e.g. by exchanging the central benzene for a naphthalene ring (as in **8**).

Even better than all the preceding modifications, however, was the introduction of a third phosphonate anion at a certain distance from the benzylic bisphosphonate (**9**). Monte Carlo simulations and NOESY experiments confirmed the postulated additional hydrogen bond contacts to all five guanidinium NH protons of the model guanidinium salt, which was now surrounded by all three phosphonate arms (Figure 7).

Strong binding and a pronounced selectivity for arginine over lysine and histidine derivatives was now observed in methanol, with K_d values in the low micromolar range (~30 μM). Monte Carlo simulations in water showed that the long trisphosphonate allows the arginine side chain to form a thermodynamically favoured extended conformation while an electrostatic interaction with the α-ammonium group still occurs. Unfortunately, affinities in aqueous solution remained small (*21*).

From the new trisphosphonate unit, we subsequently developed the first synthetic RGD receptor molecule (Figure 8). Alkylation of the deprotonated benzamide –NH group with phthaloyl-protected *m*-aminobenzyl bromide smoothly yielded **10** after hydrazinolysis and

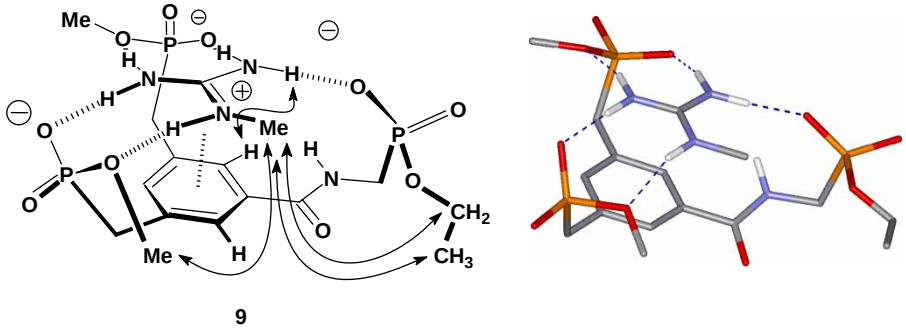
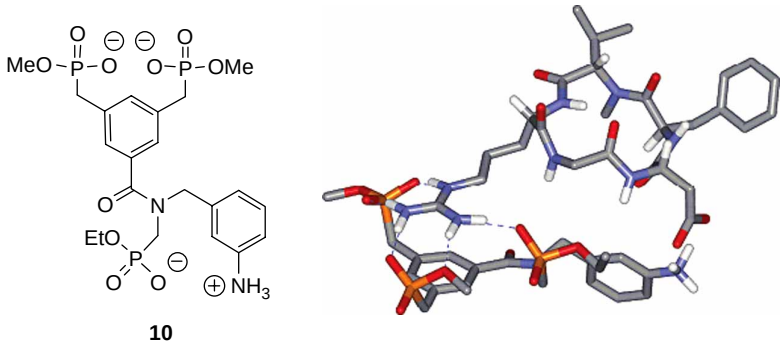


Figure 7. Summarised reciprocal NOE contacts measured between the new arginine host molecule **9** and methylguanidinium cation (500 MHz, 1:1 complex in CD_3OD). Right: Monte Carlo simulation (MacroModel 7.0, water/GBA (generalised Born approximation), Amber*, 1000 steps).



10

Figure 8. Primitive synthetic RGD receptor molecule **10** with restricted rotational freedom. Left: Lewis structure; right: molecular mechanics calculation of its 1:1 complex with cyclo(RGDfV) (MacroModel 7.0, water (GBA), Amber*, 1000 steps).

phosphonate ester cleavage with LiBr. Now a simple aspartate-binding site is present, while at the same time the rigid benzyl spacer prevents intramolecular dimerisation, and the ammonium functionality is a bad guest for the trisphosphonate and should thus circumvent an unwanted intermolecular dimerisation.

NMR titrations of the new RGD receptor molecule with the free RGD peptide as well as the integrin antagonist cRGDfV became now possible in water and furnished K_d values in the high micromolar regime. Distinct NMR signal shifts were observed for the aspartate CH_2 protons, testifying to its additional interaction with the RGD host; complex stoichiometries were determined at a precise 1:1 ratio. This showed that the concept of combining an arginine binder with an aspartate binder was valid (*22*).

Inspired by this work, the Gilson group chose our simple ditopic RGD receptor molecules and their complexes with RGD peptides as a biologically relevant model case to study the accuracy of their new programme for the calculation of absolute Gibbs energies. Their results were in good agreement with the experimental values that had been independently determined in the Schrader group. For the interaction between **10** and cRGDfV, e.g. the experimental data showed a ΔG value of -3.91 kcal/mol, which was reproduced *in silico* with -3.11 kcal/mol. A most remarkable feature of the new programme is its ability to include solvent and entropic effects in the M2 modelling method. In the course of these calculations, the Gilson group noticed that in cRGDfV-complexation by **10**, electrostatic interactions play a much smaller role than for the linear RGD peptide, while van der Waals and non-polar terms became dominating. This is in line with intuition, because cRGDfV has two charged groups less than free RGD, but carries two additional non-polar groups. The M2 calculations also suggested that much of the receptor did not interact directly with the RGD sequence. Finally, new receptor molecules were designed *in silico* and predicted to produce Gibbs energies of up to 5 kcal/mol higher than **10**. One of the calculated structures reached predicted nanomolar affinities with the cyclic RGD model peptide, rivalling protein–ligand interactions. The underlying design concept strived either to extend the hydrophobic contact area between host and guest ligand or to reduce entropic or electrostatic desolvation penalties. The only problem with these optimised virtual RGD receptor molecules was that they were too difficult to synthesise (*23*).

In an independent investigation, the influence of the spacer unit was studied with simple model systems. The benzyl group in **10** was replaced by small peptides, capable of hydrogen bonding to the backbone amides of the RGD sequence. These carried on one end a bisphosphonate and on the other end an anilinium unit. Interestingly, only short dipeptides turned out to constitute suitable spacers for RGD recognition. Obviously, the distance between arginine and aspartate recognition unit is critical for high affinity, and a better aspartate binder is needed (*24*).

In 2007, the next generation of artificial RGD receptor molecules was developed. To this end, the Schrader and Schmuck groups joined forces and connected the bisphosphonate motif with a guanidiniocarbonylpyrrole developed earlier by Schmuck. This acylguanidinium species with lowered basicity (pK $\sim$ 6–7) forms strong H-bond-enforced ion pairs to carboxylate anions; these are further strengthened by additional hydrogen bonds from the pyrrole NH and its aromatic carboxamide. *In toto*, these combined hydrogen bond donors comprise the most potent aspartate and glutamate receptors for aqueous media known to date (Figure 9). Through the combination of ion pairing with multiple ionic hydrogen bonds, these receptor molecules effectively bind to single, isolated amino acid carboxylates with millimolar K_d values in aqueous DMSO (*25*, *26*).

In order to investigate the influence of the spacer unit connecting the arginine and aspartate receptor units, four different hosts were synthesised with short peptidic linkers of varying length and rigidity (Figure 10). Compounds **11**–**13** were very well soluble in water (>1 mM), whereas the solubility of compound **14** with an aromatic spacer was still in the high micromolar range. Detailed binding studies

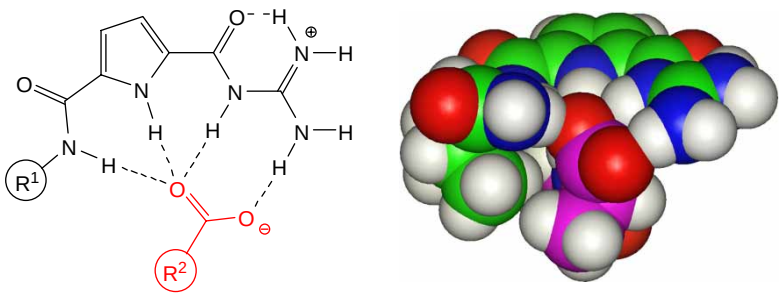


Figure 9. General structure of the guanidiniocarbonylpyrrole recognition motif and the network of ionic hydrogen bonds formed on complexation of carboxylate ions. Right: CPK model showing the calculated complex structure of Ac–RGD–NH_2 peptide (pink) bound by superior receptor molecule **14** with a guanidiniocarbonylpyrrole skeleton (green).

were carried out with NMR titrations for hosts **11**–**13** and UV–vis as well as fluorescence titrations for **14**. The pH was fixed at 6.1 (NaH_2PO_4/Na_2HPO_4 buffer), and complexation was monitored and quantitatively evaluated by standard nonlinear regression methods for the free RGD peptide as well as for N/C-protected derivatives.

No binding occurs with receptor **13**, weak complexation is found for hosts **11** and **12**. As a potential explanation, force field calculations produce stable structures for the isolated host molecules, with inter- or even intramolecular self-association between their bisphosphonate head and guanidiniocarbonylpyrrole tail. These were, in part, confirmed by dilution studies.

Obviously, the peptidic linkers built into hosts **11**–**13** are too flexible. On the contrary, no intramolecular complexation is possible in receptor molecule **14** with its rigid *m*-aminobenzoyl linker. Consequently, UV–vis as well as fluorescence titrations pointed to a significant attraction of RGD peptides by host **14**, with the N/C-protected Ac–RGD–NH_2 superior to the free RGD peptide. An attempt to probe the contribution of electrostatic interactions between bisphosphonate anion and guanidinium cation furnished a surprising result: receptor **14** with methyl esters instead of phosphonate anions

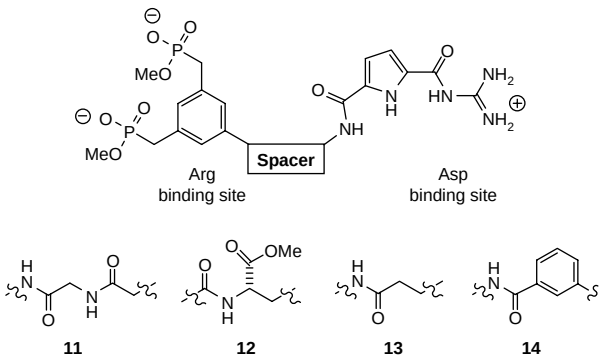


Figure 10. New generation of RGD receptor molecules **11**–**14** with bisphosphonate and guanidiniocarbonylpyrrole moieties interconnected by various spacers.

produced the tightest complex of all. This is counter-intuitive, but may well reflect differences in the solvation behaviour of both receptors. It is also in good agreement with observations from Gilson et al. (*23*) who showed earlier that Coulomb interactions between the highly solvated phosphonate anions and arginine's guanidinium cation are of limited importance, because the structure and complex stability depend more on hydrophobic interactions (vide supra) (*27*).

Conclusion and outlook

Research directed towards powerful RGD receptor molecules has been difficult and is still in progress. Several steps forward have been taken, demonstrating the need for strong arginine and aspartate binders, as well as for suitable spacers, which prevent self-association of the self-recognising complementary head groups and provide the optimal distance for complex formation of the RGD side chains without introducing any strain. Mere Coulomb attraction seems to be outperformed by non-polar interactions and hydrophobic effects. Affinities in buffered aqueous solution are still several orders of magnitude lower than those found for the RGD protein–integrin pairs. In order to bridge this gap, the Schrader and Schmuck groups are currently synthesising a third receptor generation in which the relatively weak binding and highly solvated bisphosphonate will be replaced by a much more powerful arginine binder which was discovered very recently. This is a concave belt like molecular tweezer with phosphonate tips, which binds N/C-protected arginine derivatives with low micromolar affinity in water (*28*). Interconnection of the tweezer with the guanidiniocarbonylpyrrole by an appropriate and optimised linker unit will hopefully not only lead to powerful RGD receptors but at the same time provide some selectivity for different RGD conformations, another prerequisite for a potential application as therapeutic agents.

The best candidates will be tested in bioassays for their *in vivo* properties. For major therapeutic target integrins ($\alpha_V\beta_3$, $\alpha_{IIb}\beta_3$ and $\alpha_5\beta_1$), Humphries et al. (*29*, *30*)

developed (a) solid phase protein–protein assays and (b) cell-based adhesion assays, which allow determination of IC_{50} values for cell attachment inhibition.

One day, such artificial receptors may serve as lead structures for the development of new therapeutic drugs for the treatment of diseases such as arthritis, heart attack and stroke, viral infections or cancer.

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